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13. ABSTRACT (Maximum 200 Words)

This fellowship, originally granted to Dr. M. Tsai and later transferred to Dr. H. Oketch-Rabah in the laboratory of Dr. R. Lupu, was initially concerned with the role of the cytokine heregulin, in the regulation of hormone receptor status in breast cancer. Three projects were accomplished during the fellowship. The first based on the original SOW yielded information that demonstrated that Cyr61 is a downstream effector of Heregulin (HRG) action and suggested that Cyr61 is necessary for HRG-mediated chemomigration. Furthermore the work showed that Cyr61 plays a functional role in breast cancer progression, possibly through its interactions with the alpha(v)beta3 receptor (see attached publications).

The findings of Dr. Oketch-Rabah on the Black Cohosh project demonstrated that BC has no estrogenic activity and therefore is safe to use for the treatment of menopausal symptoms by women at risk of developing breast cancer and in whom estrogen replacement therapy is contraindicated for other reasons (see attached Abstracts).

In the final year we requested a modified SOW due to Dr. Lupu's relocation. Dr. Oketch-Rabah began a project in the Barcellos-Hoff laboratory to examine TGF-β1 regulation of the proliferation of estrogen receptor positive (ER+) cells during mouse mammary development. To our knowledge this is the first mouse model to demonstrate a mechanism controlling the size of the ER+ subpopulation and a means of investigating how the dysregulation of this population contributes to the risk of developing breast cancer (see attached Abstracts).

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The Role of TFG-β1 in the Regulation of Estrogen Receptor During Mouse Mammary Development and Carcinogenesis

INTRODUCTION

This final report covers the entire project period for this grant dating from August, 1999 to 2003 and includes the work that was done by Dr. M. Tsai, the original grantee whose research dealt with "The Involvement of Human Cyr61 in Heregulin Induction of Breast Tumor Progression" under the original SOW (SOW1 attached) and the work done by myself, the current grantee, Dr. Hellen Oketch-Rabah. I have been supported by the project since December, 2001. Under the mentorship of Dr Lupu I investigated the "Estrogenic Activity of Black Cohosh-BC" from December 2001 until May 2002 when Dr. Lupu left LBNL. Thereafter, under the mentorship of Dr. Mary Helen Barcellos-Hoff, we requested a new SOW (SOW2 attached) to investigate the "Role of TFG-β in the Regulation of Estrogen Receptor During Mouse Mammary Development and Carcinogenesis".

Although it is not conventional, for the convenience of the reader, the main body of this report will be divided into 3 Sections (A-C), describing the three projects that were done during this fellowship.

Section A: The overall goal of the Cyr61 project was to determine the role of Cyr61 a secreted protein that induces proliferation of blood vessels and is also an adhesion molecule in cell-cell interaction, in breast tumor progression, invasion and spread. Specifically these studies were designed to test whether Cyr61 acts as a stimulator in breast cancer cells and assist tumors to grow and spread by attracting new blood vessels and to test whether inhibition of Cyr61 activity would cut blood supply, stop tumor spread and even shrink the tumors. The research made use of *in vitro* methods namely immortalized human epithelial cell cultures and *in vivo* methods in which immunodeficient mice were implanted with human breast cancer cells.

Section B: The Black Cohosh project was part of an ongoing research project in the laboratory of Dr. Ruth Lupu to investigate the estrogenic activity of *Actaea racemosa* L., commonly known as Black Cohosh. The goal was to evaluate the estrogenic activity of several pytomedicines that were either about to be introduced or already in the market. BC was one of the phytomedicines under study. The specific objective was to determine whether extracts from this plant had any estrogenic activity that would present a risk to women breast cancer patients with ER+ breast cancer or those in remission or at risk of developing ER+ breast cancer for other reasons. Analysis was done on aqueous and organic extracts using cell based and molecular assays that investigated whether these extracts had any estrogen-like activity at cellular and message level.

Section C: The estrogen receptor (ER) project in the laboratory of Dr. Mary Helen Barcellos-Hoff investigated a mechanism that regulates the ER+ mammary epithelial cell population. The objective was to determine the role of transforming growth factor-β1 (TGFβ1) in the regulation of the ER population. We made use of a transgenic mouse model that lacks one TGFβ1 allele and consequently has only 10 % equivalence of the level of TGFβ1 found in the *Tgf-β1* (+/+) mice.

BODY

Section A: The Involvement of Human Cyr61 in Heregulin Induction of Breast Tumor Progression.

The first grantee Dr. Tsai showed that Cyr61 is necessary for HRG-mediated chemomigration and plays a functional role in breast cancer progression, possibly through its interactions with the alpha(v)beta3 receptor (1). She further demonstrated that Cyr61 promotes acquisition of estrogen-independence and anti-estrogen resistance *in vivo* in breast cancer cells and also that Cyr61 induces tumor formation and tumor vascularization *in vivo* through events mediated via the activation of the MAPK and the Akt signaling pathways. They investigated how Cyr61 expression is regulated in both estrogen receptor positive and (ER+) and estrogen receptor negative (ER-) breast cancer cells and demonstrated that Cyr61 mRNA and protein expression is inducible by estrogen and anti-estrogens in ER-positive breast cancer cells, that a labile protein as well as a negative regulator might be involved in Cyr61 expression in estrogen-dependent breast cancer cells. Other important regulators of Cyr61 expression in breast cancer cells that they found included TPA, vitamin D, and retinoic acid. Interestingly, most of these effects were not seen in aggressive breast cancer cells that do not express ER and express high levels of Cyr61, such as the MDA-MB-231 cells. They suggested an association between increased Cyr61 expression and an aggressive phenotype of breast cancer cells (2,3).

Section B: Estrogenic Activity of Black Cohosh (BC).

In the second year of the project, Dr. Tsai left LBNL for another position and I joined the laboratory of Dr. Ruth Lupu to conduct research on *Actaea racemosa* L., commonly known as Black Cohosh and herein referred to as BC. Extracts of roots/rhizomes of this plant are taken as a remedy to alleviate menopausal symptoms, such as hot flashes and is available in various formulae over the counter to women as an alternative to estrogen replacement therapy. It is claimed to reduce the frequency of hot flashes. However, the mechanism by which it does so is still unknown. There are conflicting reports in the literature showing that BC has estrogenic activity and anti-estrogenic activity. The overall goal of that project was to evaluate natural products (herbal medicines/phytomedicines) that like BC were either already in the market or about to be introduced for alternative treatment of breast cancer and menopausal symptoms [specifically for women in whom estrogen replacement therapy is contraindicated because they have other risk factors for breast

cancer]. To achieve this goal extracts (hexane, ethyl acetate, and aqueous) were prepared from BC by sequential solvent partitioning. These were tested for estrogenic activity using the Ishikawa cell assay that measures the estrogenic activity of compound(s) by inducing an endogenous alkaline phosphatase (AP) enzymatic activity in the Ishikawa cell line, a non-radionactive cell proliferation assay ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS assay) that determines the extracts' effects on the *in vitro* growth of MCF-7 and MDA-MB-231 breast cancer cells and using the soft agar assay to assess the effects on the anchorage-independent growth of breast cancer cells. At the molecular level, the ability of the BC extracts to modulate the estrogen receptor (ER) function was evaluated using the estrogen-responsive element (ERE)-luciferase reporter assay.

BC extracts neither induced the transcriptional activation of ERE, nor regulated the expression of estrogen-regulated genes in the RNase protection assay. These extracts showed no effect on the growth of ER-positive breast cancer cells and did not induce colony formation in ER-positive cells. These data corroborated preliminary data that had earlier been obtained in the lab and together all the data demonstrated no estrogen-like activity in any of the BC extracts. We therefore concluded that BC roots and rhizomes are safe for use as an herbal remedy for the treatment of hot flashes in menopausal women for whom estrogen replacement therapy would be contraindicated. A manuscript based on these results has been accepted for publication in the International Journal of Oncology (see attached e-mail message from Dr. R Lupu, and an original abstract of the manuscript).

Section C: Role of TFG-β in the Regulation of Estrogen Receptor During Mouse Mammary Development and Carcinogenesis

Dr. Lupu left LBNL in May 2002. The final year of the project was spent doing research with Dr. Mary Helen Barcellos-Hoff at LBNL investigating the mechanisms involved in the regulation of the ER positive cells during normal mouse mammary gland development. Although the project initiated by Dr. Tsai in Dr. Lupu's lab was based on the hypothesis that ER+ cells switch to ER- during human mammary carcinogenesis, it is debated whether such a switch actually occurs. Recent literature reports suggest that ER+ and ER- tumors may actually arise via different mechanisms (4). Therefore our interest is to understand the regulation and maintainance of the ER+ mammary gland population during normal mouse mammary gland development. Our view is that an in-depth knowledge in this area will enable us to identify potential mechanisms regulating the frequency of ER+ cells and how dysregulation may occur during carcinogenesis.

The processes of mammary gland development, differentiation and neoplasia are fundamentally regulated by the sex steroids, progesterone and estrogen (5). It is well known that estradiol (E2) signaling through ER-α (one of the isoforms of ER) plays a central role in mammary epithelial cell proliferation while progesterone signaling mediated via the progesterone receptor is mostly involved in the process of secretory differentiation in the mammary gland together with prolactin signaling during pregnancy (6). **Increased frequency of ER+ve cells is associated with breast cancer risk (7-9) and ER positive breast cancer is postulated to arise by a distinct pathway from ER negative tumors (4).** Unexpectedly, several studies have demonstrated that the majority of ER+ cells do not proliferate, but instead regulate proliferation in ER-negative (ER-) cells via a paracrine mechanism (10-13). It has for a long time been postulated that the action of these hormones that is via the receptors is through the induction of certain growth factors that regulate proliferation.

Previous studies in the Barcellos-Hoff lab had shown that TGF-β1, which is a cytokine that is implicated as both a tumor suppressor and a tumor promoter in human breast cancer, is differentially regulated by estrogen and progesterone, and in turn mediates mammary growth (14). TGF-β1 is a pluripotent cytokine that is widely implicated in regulating mammary epithelial growth (15-18). TGF-β1 can cause profound inhibition of epithelial proliferation, induction of apoptosis and extracellular matrix deposition and remodeling. A variety of studies support the idea that TGF-β1 can act via autocrine, paracrine, and endocrine mechanisms of action (reviewed in 19).

It has been previously been suggested that ER + cells, in response to estrogen stimulation, produce a growth factor that stimulates the epithelial cells to proliferate (20-21). However studies in humans, rat and mouse have shown that most ER+ cells do not proliferate (10) and hence the question arises as to what restrains the non-proliferating ER+ cells from responding to the growth factor that they produce?

Mammary epithelial cell proliferation is dictated by the endogenous hormonal milieu such that epithelial proliferation peaks at estrus. Previous work in the Barcellos-Hoff lab using C57/BL-129SV had shown that the extent of proliferation in *Tgfβ1* +/+ mice and *Tgfβ1* +/- mice correlated with estrus cycle stages (14). Furthermore at estrus when there is a high rate of cell proliferation, nearly all ER+ cells co-localized with intense TGF-β1 staining, consistent with their non-proliferative status (Ewan, Oketch-Rabah and Barcellos-Hoff, unpublished data). We therefore hypothesized that TGF-β1 acts as a brake restraining the ER+ cells from proliferating while at the same time the ER+ cells, in response to hormonal stimulation by estrogen, send out a signal to the ER- cells to proliferate.

Research in the final year of this fellowship was therefore designed to determine the relationship between TGF-β1 and ER+ cells in the Balb/c mouse mammary epithelium. We hypothesized that decreased TGF-β1 levels would lead to an increased proliferation of ER+ cells in the mouse mammary epithelium.

In order to investigate this hypothesis we formulated two aims for the final year of this fellowship as follows:

Aim 1: To substantiate the role of TGF-β1 in regulation of ER during mammary development.

Aim 2: To determine whether TGF-β1 suppresses ER using primary mouse mammary epithelial and human breast cell cultures.

Aim 1 has been adequately addressed and in fact has provided answers that we had expected to obtain by pursuing **Aim 2**.Consequently I concentrated all efforts on Aim 1.

RESULTS

Aim 1: To substantiate the role of TGF-β1 in the regulation of ER during mammary development.

To determine the proliferative index, cryosections of glands from adult nulliparous Balb/c heterozygote Balb/c mice were stained with a rabbit polyclonal antibody to Ki67 and the nucleus counterstained with 4,6-diamidino-2-phenylindole (DAPI) nuclear dye. Cells immunoreactive to Ki67 were scored and the index of proliferation determined as a percentage of the total number of luminal epithelial cells staining with DAPI nuclear dye.

***Proliferation is increased in TGF-β1* (+/-).**

Table 1: Proliferation is altered and the proliferating ER+ cell index increase significantly in adult *TGF-β1* heterozygote mice.

	Colum2 ***%Ki-67+***		***Colum3*** ***% ER-α+***		***Colum4*** ***% ER-α Ki-67+*** [a]	
Estrus Stage	*Tgfβ1* (+/+) wildtype	*Tgfβ1*(+/-) heterozygote	*Tgfβ1*(+/+) wildtype	*Tgfβ1*(+/-) heterozygote	*Tgfβ1*(+/+) wildtype	*Tgfβ1*(+/-) heterozygote
Proestrus	2.9	2.5	30.0	32.0	0.0	0.0
Estrus	9.3	12.0*	32.0	37.0	1.5	3.5*
Diestrus	2.3	9.8*	29.1	34.0	0.5	2.8

[a] At least 30 fields from 3 animals were scored for frequency of cells with ER/Ki67 positive nuclei. Total number of cells were determined by DAPI counterstain (number positive/total epithelial cells x 100). [b] Significant difference ($p<0.05$)* from *Tgf-β1* (+/+) mice using Fisher's exact test.

I found that proliferation was increased in and *Tgfβ1* +/- mice compared to *Tgfβ1* +/+ mice. Both genotypes exhibited the maximum number of Ki67 positive cells at estrus. However, at estrus the proliferative index was 1.3 fold greater in *Tgf-β1* (+/-) compared to *Tgf-β1* (+/+) littermates (Table 1, column 2). Similar results had earlier been obtained in Barcellos-Hoff lab in C57/bl/129SV in which proliferation increased by 4-fold in *Tgfβ1* +/- mice compared to *Tgfβ1* +/+ animals at estrus. A significantly higher Ki67 index was also observed during diestrus in the *Tgf-β1* (+/-)

compared to the *Tgf-β1* (+/+) animals. Thus, proliferation persisted during diestrus in the Balb/c *Tgfβ1* +/- mice but not in the *Tgf-β1* +/+. This was not the case in mouse bred to the C57BL/129SV background (data not shown) in which the number of proliferating cells decreases to levels similar to that found at proestrus in both genotypes. Thus the phenotype of the *Tgfβ1* +/- mice depends on the hormonal status of the adult animal.

The lower indices of proliferation reported by Ewan *et al.* 2002 (14) are attributable to the fact that the: Proliferating cell nuclear antigen (PCNA) proliferation index used in those studies labels only cells at the S phase during proliferation, while the Ki67 used in the present studies labels cells in cycle at the G1, S, G2 and the M phase. Thus Ki67 immunoreactivity covers a wider window in the proliferation cycle and thus cells at different stages of the cycle are labeled giving a larger number of cells in cycle than the PCNA.

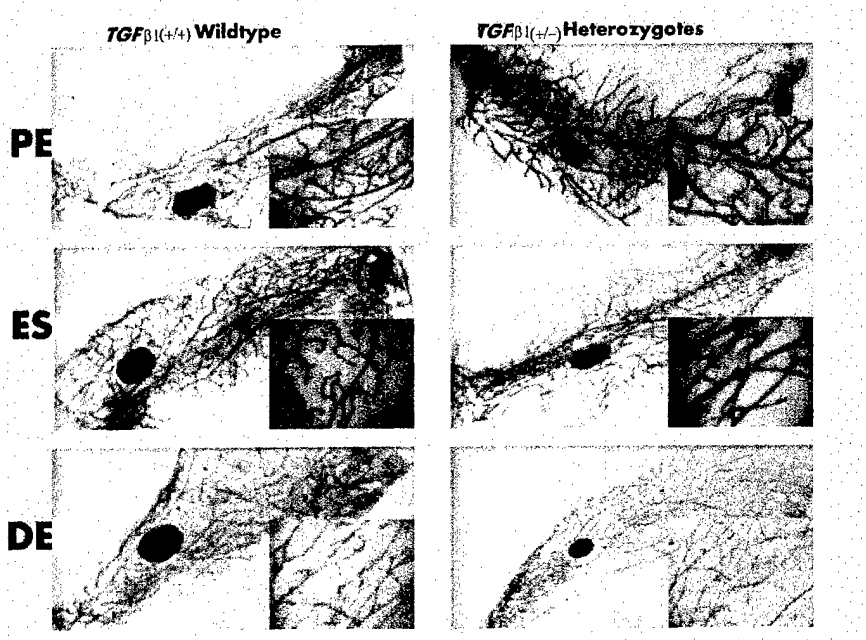
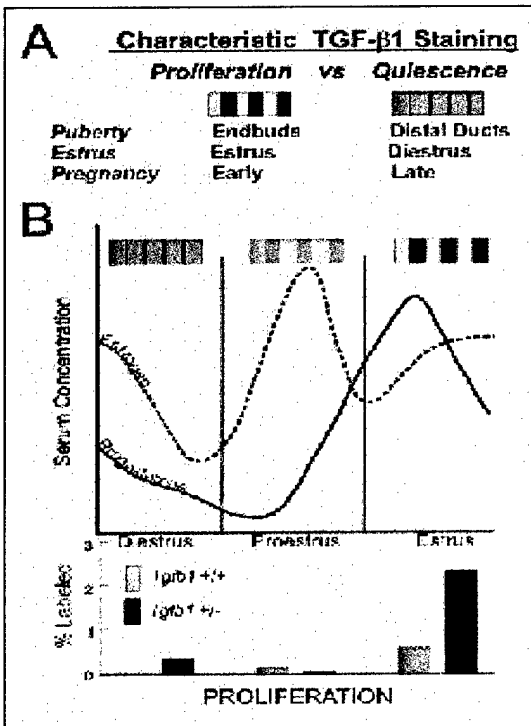


Figure 1: Whole mounts of Balb/c *TGF-β1 (+/+)* and *TGF-β1 (+/-)* mice at different stages of the estrus cycle (PE-proestrus, ES-estrus, DE-diestrus) stained with carmine alum.

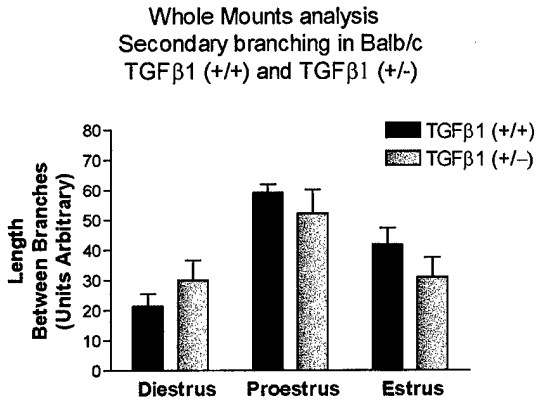
As in C57BL/129SV we observed that mammary whole mounts of Balb/c mice were normal (Figure 1). The lack of hyperplasia in the tissue of *Tgfβ1* +/- mice appears to be due to an apoptotic homeostasis mechanism (14). To confirm this, I also analyzed whole mounts of mammary glands obtained from *Tgfβ1* +/- and *Tgfβ1*+/+ Balb/c mice at different stages of the estrus cycle and stained

with alum carmine. Although the glands from both *Tgfβ1* +/- and *Tgfβ1*+/+ developed normally and over all the glands appeared similar (Figure 1) there were subtle differences in the branching pattern at different stages of the estrus cycle that we quantitated.

Using SigmaScan software (SPSS, Chicago IL) we determined the distance between branches on a secondary branch in images of these glands. The total distance of each branch measured was the same, in all animals, therefore these figures translates to the largest number of branches in both genotypes being at diestrus and the fewest at proestrus, while a intermediate value between the two extremes is found at estrus. The mean length between branches at different stages of the estrus cycle was significantly different between the different stages of the estrus cycle in the *Tgf-β1* (+/+) mice. However there was no significant difference between the *Tgf-β1* (+/-) and *Tgf-β1* (+/+) Balb/c mice. The length between branches was longest at proestrus and shortest at diestrus



Reprinted from Ewan *et al.* 2002



C Branching pattern at different stages of the estrus cycle in *Tgf-β1* (+/+) and *Tgf-β1* (+/-) Balb/c mice.

Figure 2: A Characteristic TGF-β1 staining during proliferation and quiescence at puberty, during, estrus and pregnancy in *Tgfβ1* +/- and *Tgfβ1* +/+ Balb/c mice.

B Hormonal changes during the estrus cycle as correlates with proliferation in *Tgfβ1* +/- and *Tgfβ1* +/+ Balb/c mice.

C Distance between branches in on a secondary branch at different stages of the estrus cycle.

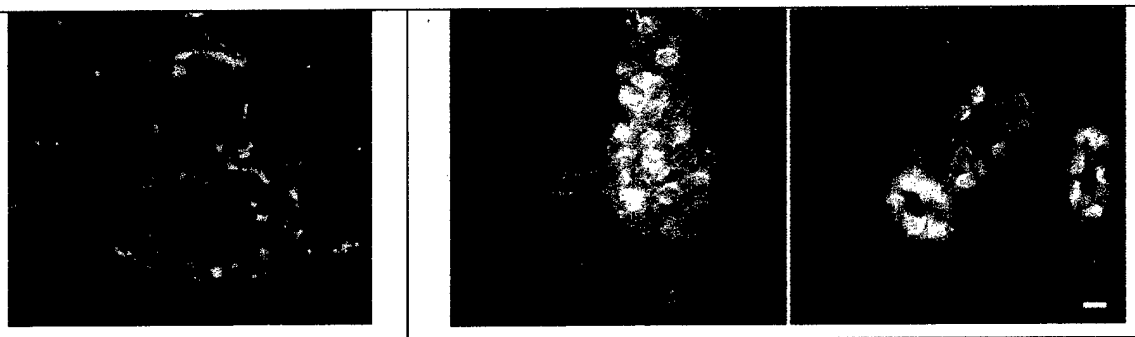
In the *Tgf-β1* (+/+) mice, significantly more branches are observed in animals that were at estrus compared to diestrus while in the *Tgf-β1* (+/-) mice the mean number of branches is similar at estrus and diestrus. These observations are consistent with the fact that proliferation is highest during and immediately after the upsurge of estrogen, while formation of side branches corresponds to periods of progesterone upsurge and immediately thereafter. However as the estrogen and progesterone levels begin to decrease at diestrus, the tiny side branches that formed in preparation for pregnancy and lactation regress. Thus at proestrus there is apparent increase in length between branches as the tiny branches that had earlier formed at estrus will be absent.

Thus far these findings supported our hypothesis that TGF-β1 restrains mammary epithelial cells from responding to hormonal stimulus to proliferate. Whereas the proliferation index was similar in both *Tgfβ1* +/+ and *Tgfβ1* +/- Balb/c mice at proestrus, there was a 4.8 fold increase in proliferating cells at estrus in the *Tgfβ1* +/- mice compared to only a 3.3 fold increase in the *Tgfβ1* +/+ mice, indicating that the *Tgfβ1* +/- mice exhibit a stronger proliferative response to the hormonal upsurge preceding the estrus stage. This resulted in more proliferating cells in the *Tgfβ1* +/- mice compared to the *Tgfβ1* +/+ mice.

TGF-β1 Inhibits Proliferation of Estrogen Receptor Positive Mammary Epithelial Cells:

In order to understand the dynamics of the ER+ epithelial cell population, sections of mammary epithelium from adult nulliparous Balb/c mice were stained with a mouse monoclonal antibody to ERα (NCL-ER-6F11) and rabbit polyclonal antibody to Ki-67. We compared the pattern of heterogenous staining of the ER+ cells at estrus (Figure 3a) with the pattern of staining of active TGF-β1 that had earlier been demonstrated in Mary Helen Barcellos-Hoff lab (Figure 3 b,c) and found that they were quiet similar. Further more in the earlier studies dual staining of sections of mammary gland collected at estrus for TGF-β1 and ER had shown that 22% of cells were ER+ in the Balb/c strain and 18.8% were positive in the C57Bl/6-129SV strain.

The observation that ER+ cells and TGF-β1 positive cells were heterogeneously distributed at estrus and colocolized in about 20% of the cells indicated that a group of ER+ cells were activating TGF-β1 at estrus. However TGF-β1 can be activated in an autocrine or paracrine fashion and therefore we sought to investigate whether TGF-β1 signaling pathway is activated in the ER+ cells themselves or in neighboring cells.



a) ER+ cells nucleus	b), c) LAP (green and homogenous) and Active TGF-β1 (shows as yellow due a mixture of red and green)

Figure 3: a) Balb/c mouse mammary gland at estrus: ER+ cells nuclei (green and heterogenous), other nulei stained with DAPI only are blue.
b) & c) Latent TGF-β1 activation in mammary gland adapted from Ewan et al., *Am J Path.* 160:2081-93 2002)

We performed dual staining of ER and Smad2/3 (shown in Figure 4b). TGF-β signaling begins with ligand binding to its serine-threonine kinase receptors. The type II receptor (TβR-II) forms a heterodimeric complex with, and phosphorylates, the type I receptor (TβR-I), which in turn phosphorylates two cytosolic proteins, Smad2 and Smad3 (R-Smads). Once activated these proteins form a complex with another cytosolic protein, Smad4 and translocate to the nucleus, where they bind to DNA and regulate gene transcription. Therefore translocation of Smad2/3 to the nucleus indicates activation of TGF-β pathway.

If TGF-β1 biological activity is restricted to certain cells of the epithelium we would expect to see variation in the nuclear localization of R-Smad. Indeed we found that R-Smad is present in the cytoplasm of many epithelial cells but is localized in the nucleus of only a subset. A majority of this subset was also positive for ER. Only a few of the ER+ cells (<1%) had no R-Smad translocated to the nucleus. This is a clear indication that the TGF-β1 pathway is activated in the ER positive cells and supports our hypothesis that TGF-β1 restrains these cells.

Since almost all ER+ mammary epithelial cells activate TGF-β1, we wished to determine the consequences of TGF-β1 activation on proliferation in this cell subpopulation. We hypothesized that depletion of TGF-β1 would result in an increase in proliferating ER+ positive mammary epithelial cells and, consequently, an increased frequency of ER+ positive cells in the mammary epithelium.

Accordingly, we scored epithelial cells in mammary gland sections from *Tgfβ1*+/+ mice and *Tgfβ1* +/- mice obtained at diestrus, proestrus and estrus for immunoreactivity of ER and the cell cycle marker Ki-67. Three animals per each estrus stage and a minimum of 10 ducts for

each animal were evaluated for each group (Table 1, column 3 and 4). We found that the frequency of ERα cells in cycle was significantly increased ($P<0.05$, t-test) from 1.5% in *Tgfβ1*+/+ mice to 3.5% in *Tgfβ1*+/- mice. TGF-β1 depletion results in an increased frequency of both Ki-67 positive and dual positive (ERα positive/Ki-67 positive) mammary epithelial cells at estrus, which suggests that the latter is a constant proportion of the former. However this was not the case for the different hormonal environment at proestrus. At this stage in Balb/c mice, the frequency of cells positive for Ki-67 in mammary epithelium was 2.5% but there were no dual positive cells in either the *Tgfβ1*+/+ mice or in the *Tgfβ1*+/- mice .

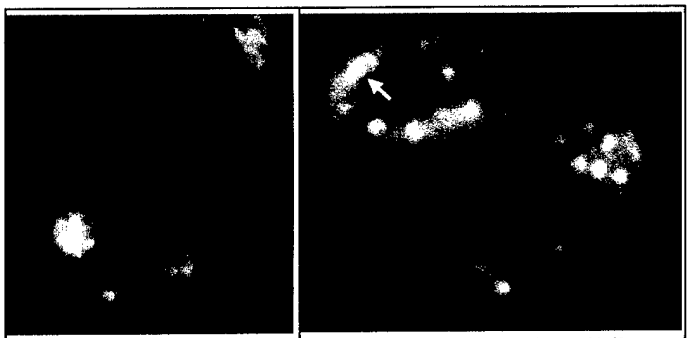


Figure 4: a) ER & Ki67 | **Figure 4:** b) ER & Smad2/3

a) ER (green) & Ki67 (red) colocolized (yellow)
b) ER (green) & Smad2/3(red) colocolized (yellow)

A consequence of increased frequency of ERα positive mammary epithelial cells in cycle should be accumulation of these cells. We therefore determined the frequency of ERα cells in the mammary epithelium of nulliparous Balb/c mice. ER+ cell frequency increased significantly ($P<0.05$, t-test) at all stages of the estrus cycle in the *Tgfβ1* +/- mice when compared to the *Tgfβ1* +/+ littermates (Table 1, column 4.

DISCUSSION

It is already well established that TGF-β1 can profoundly inhibit mammary epithelial cell proliferation and morphogenesis (15-18). However it is not known when this action occurs in normal breast or how it is regulated. The activity of TGF-β1 is restrained by its production by all cells in a latent complex. TGF-β1 needs to be released from this complex in an extracellular process called activation in order to influence cells (22). Previous studies in the Barcellos-Hoff laboratory revealed that certain antibodies recognize TGF-β1 only upon its release from the latent complex, i.e. following activation while other antibodies recognize the TGF-β1 bound to the latent associated peptide (LAP). Thus it was possible to distinguish cells in which TGF-β1 is active from those cells in which TGF-β1 is inactive (23).

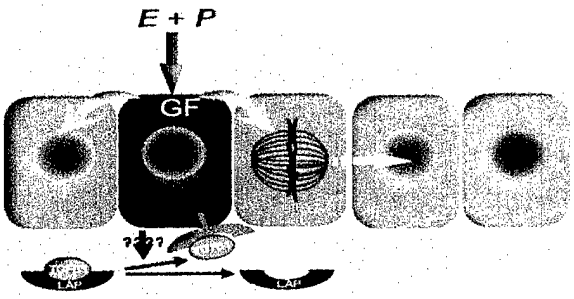


Figure 5: Schematic showing activation of TGF-β1 in ER+ cells in response to increased levels of E+P

There are three general consequences of TGF-β1 activation: inhibition of DNA synthesis, induction of apoptosis and modulation of ECM remodeling. Earlier studies in the Barcellos-Hoff laboratory published in the American Journal of Pathology (14), showed that *TGF-β1 production and activity are differentially regulated by ovarian hormones, estrogen and progesterone* and that *TGF-β1, in turn, regulates mammary development via its effects on apoptosis and proliferation.*

We studied the dynamics of the ER+ epithelial cell population in adult nulliparous Balb/c *Tgf-β1* (+/-) Balb/c mice. As in previous studies reported in C57Bl/6-sv 129 (14), we found that ER+ cells rarely proliferate. Although proliferating ER+ cells are most frequent at estrus in both the *Tgfβ1* +/+ and *Tgfβ1* +/- mammary epithelial population, significantly more are found in *Tgfβ1* +/- when compared to the *Tgfβ1* +/+ mice. Furthermore we observed a higher frequency of ER+ cells in both C57Bl/6-sv 129 and Balb/c *Tgfβ1* +/- mouse strains when compared to their respective *Tgfβ1* +/+ littermates.

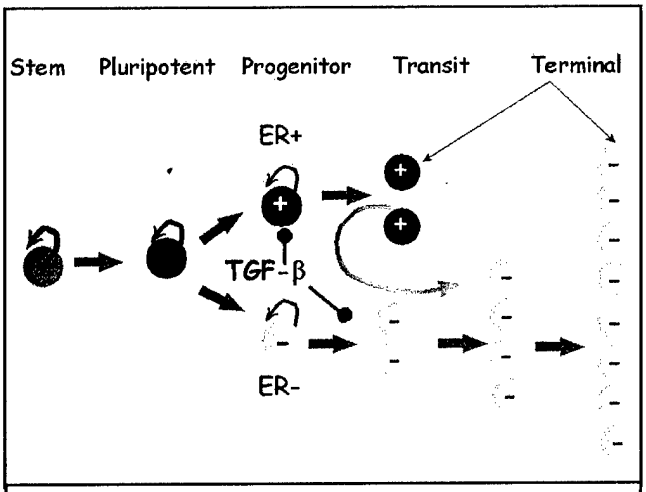


Figure 6: A Model of TGF-β1 inhibition of proliferation of ER+ progenitor cells and ER- transit cells

From these findings we concluded that reduced levels of TGF-β1 leads to increased proliferation of ER+ cells and results in an accumulation of ER+ mammary epithelial cells. This is the first mouse model to our knowledge to demonstrate a mechanism controlling the size of the ER+ subpopulation and a means of investigating how dysregulation of this population contributes to risk of developing breast cancer.

As an explanation for our observations we propose a model in which latent TGF-β1 is activated in ER+ cells when the hormones estrogen and progesterone levels reach a certain threshold. The ER+ cells then release a growth factor that informs neighboring ER- cells to proliferate but are prevented from responding themselves by TGF-β1 signaling and downstream events. A future research question is what

mechanism is involved in the actual activation of the TGF-β1 (represented in the schematic in **Figure 5** by the four question marks) in the ER+ cells.

Taken together our observations also suggests that TGF-β1 not only regulates the proliferative potential of ER+ cells, but that the ER+ cells are indeed capable of proliferating, and further implies that an ER+ cell begets an ER+ cell (hence their accumulation when the TGF-β1 restraint is removed). Our next studies will test the hypothesis, shown in **Figure 6** that ER+ cells are a separate, self-renewing cell population in the mammary epithelium. We hypothesize that the ER+ population is a pluripotent progenitor of stem cells giving rise to two populations; one that is involved in regulating proliferation in the mammary gland but themselves do not simultaneously proliferate and a second small subpopulation (about 1%) that is responsible for maintaining the ER+ epithelial mammary gland population. There is a third compartment of epithelial cells comprising the ER+ and ER- cells that are at rest (not proliferating). These transit cells have to down-regulate TGF-β1 activation to respond to the signal from ER positive cells and are also restrained from proliferating by activation of the TGF-β1 pathway as evidenced by increase in proliferation of ER- cells in the *Tgfβ1* +/- mice.

Results for Aim 2: To determine whether TGF-β1 suppresses ER using primary mouse mammary epithelial and human breast cell cultures.

The *in vivo* data that was gathered as described above demonstrated clearly that ER+ cells are suppressed by TGF-β1. Consequently we deemed it unwise to carry on with the primary mammary epithelial cells cultures that would require a very large number of animals and yet would not add to our findings significantly. However we are still committed to furthering studies using the primary epithelial cell cultures to answer or confirm specific mechanism questions once we fully understand this model.

This data was presented as a poster at the Gordon Research Conference 2003, Mammary Gland Biology (see attached abstract) and is currently being prepared for publication.

MATERIALS AND METHODS

Mice

All experiments were conducted with institutional review and approval. Animals were euthanized by CO2 inhalation and cervical dislocation at the indicated times in accordance with AAALAC guidelines. Mammary glands were collected from *TGF-β1* +/- and *TGF-β1* +/+ mice bred in house in Balb/c purebred background mice originally obtained from Adam Glick (NCI). Estrus was determined by cytological characteristics of vaginal smears and confirmed postmortem by uterine wet weight. Nulliparous animals were euthanized in estrus around 10 weeks of age. At least three animals of the same genotype were used for each treatment group and samples were collected at proestrus, diestrus and estrus. The inguinal (fourth pair) mammary glands were dissected free of the skin and either placed in Carnoy's solution or embedded in OCT compound (Miles Inc., Elkhart, IN). Frozen tissue blocks were stored at -70°C until the time of sectioning. Whole mounts staining of glands with carmine alum was done as previously described by (except that the glands were fixed in carnoy's fixative overnight, stained glands were stored in methyl salicylate after post-staining dehydration (24).

Antibodies

Active TGF-β1 was detected using polyclonal, affinity-purified chicken anti- TGF-β1 (AF-101-NA, lot FS08; R&D Systems, Minneapolis, MN), which preferentially reacts with the active form of TGF-β1. We used monoclonal antibody NCL-ER-6F11 to ERα and rabbit polyclonal antibody to Ki-67 both from Novacastra (Newcastle, UK). Antibody FL-425 (#SC-8332) recognizes several Smad proteins and was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For secondary antibodies, we used Alexa 488 labeled goat anti mouse IgG (Molecular Probes, Eugene, OR) FITC-labeled anti-mouse (Pierce, Rockford, IL), Texas red labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and Texas red labeled rabbit anti-chicken IgY (Sigma, St. Louis, MO).

Immunohistochemstry

Frozen embedded mammary glands were sectioned (3 μm) onto gelatin-coated coverslips, then fixed using 2% buffered paraformaldehyde for 20 minutes at room temperature, followed by a 0.1M glycine in phosphate-buffered saline (PBS) washes to quench the paraformadehyde. For ERα colocalization with Ki-67 or Smad sections were treated with pre-warmed 0.1% Triton X100 in PBS at 37°C for 20 minutes followed by PBS washes. Nonspecific sites were blocked using the

supernatant from a 0.5% casein/PBS solution (pH 7.4) for 60 minutes. Endogenous mouse IgG was blocked in 1:1 Mouse-on Mouse blocking agent (Vector Laboratories, Burlingame,CA) to 0.5% casein/PBS solution for 4 hours at room temperature. Sections were incubated in primary antibodies in 0.5% casein/PBS solution overnight at 4°C. After washes, each primary was detected by sequential incubations with species-specific secondary antibodies. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The sections were mounted in Vectashield (Vector Laboratories, Burlingame,CA) and stored at -20°C until evaluated. After washes, each primary was detected by sequential incubations with species-specific secondary antibodies.

Microscopy and Image Analysis

Immunofluorescence images were obtained and processed as previously described (14). Color images of ducts were split into their constituent channels and scored for total and positive cells using the text annotation feature in Corel Photopaint 7 (Corel, Dallas TX) without knowledge of tissue source of images. Two tailed Student t-tests were used to evaluate whether mean frequency values differed significantly, using Prism 3 (GraphPad Inc, San Diego CA). Intensity measurements of steroid receptor immunoreactivity in positive mammary epithelial nuclei were measured using SigmaScan software (SPSS, Chicago IL) and the measurements in each image were corrected for background. Measurements of LAP and active TGF-β1 intensity were taken as described previously (14).

KEY RESEARCH ACCOMPLISHMENTS

- Used immunohistochemistry techniques to determine the locolization and distribution of estrogen receptor, Ki67, Smad2,3, activeTGF-β1 and latent TGF-β1 in the mammary gland
- Learnt to take care of and use transgenic mice models for mammery gland studies
- Learnt to obtain and process animal tissues for protein analysis and immunohistochemistry studies
- Learnt various techniques in biochemistry and molecular biology.

REPORTABLE OUTCOMES

Publications:

- Tsai MS, Shamon-Taylor LA, Mehmi I, Tang CK, Lupu R. Oncogene. 2003 Feb 6;22(5):761-8. (copy attached).
- Tsai MS, Bogart DF, Castaneda JM, Li P, Lupu R. Oncogene. 2002 Nov 21;21(53):8178-85. (copy attached).
- *In Press*: BC Paper in International Journal of Oncology (abstract attached)
- Manuscripts in Preparation: ER paper (abstract attached)

Conferences/Meetings

- Abstract No 134: Titled "Black Cohosh *(Cimicifuga racemosa)* does not have any estrogenic activity" (attached as Appendix I) for a poster presented at the International Scientific Conference on COMPLEMENTARY ALTERNATIVE & INTERGRATIVE Medicine Research that took place in Boston in April 12-14, 2002 at the Boston Marriot Copley Place, Boston MA. (abstract attached).
- A poster presented at a post doc day meeting at LBNL titled "Transforming Growth Factor-b1 Regulates the Proliferation of Estrogen Receptor Positive Mammary Epithelial Cells"Hellen A. Oketch-Rabah, Kenneth Ewan, G. Shyamala and Mary Helen Barcellos-Hoff.Department of Cell and Molecular Biology, Ernest Orlando Lawrence Berkeley National Laboratory , Berkeley, California 94720.
- Gordon Research Conference meeting. Mammary Gland Biology Meeting held at Roger Williams University RC meeting 06/01/2003 - 06/06/2003. Abstract Title "Transforming Growth Factor-β1 Regulates the Proliferation of Estrogen Receptor Positive Mammary Epithelial Cells *Hellen A. Oketch-Rabah, *Kenneth Ewan, Shraddha Ravani, *Shyamala G. and *Mary Helen Barcellos-Hoff

PROPOSAL:

- Results of this research formed part of a proposal that is now funded by NIH to further investigate the ER+ mammary epithelial population in mice.

CONCLUSIONS

This fellowship has been unique in that it has supported two post-doctoral fellows and a wide range of research spanning normal breast development to carcinogenesis in the breast and to phytotherapeutics. The common thread in all these research projects has been the quest for a better understanding of the role and regulation of ER+ cells in the mammary gland.

The findings of the initial project on Cyr61 yielded information that demonstrated that Cyr61 is a downstream effector of (Heregulin) HRG action and suggested further that Cyr61 is necessary for HRG-mediated chemomigration. Further the work showed that Cyr61 plays a functional role in breast cancer progression, possibly through its interactions with the alpha(v)beta3 receptor (1).

The findings of the Black Cohosh project demonstrated that BC has no estrogenic and therefore is safe by women at risk of developing breast cancer and in whom estrogen replacement therapy is contraindicated (see attached Abstract).

The ER project in the Barcellos-Hoff laboratory has elucidated an important mechanism that is involved in controlling the ER+ epithelial cell population during normal mouse mammary development. We have shown that TGF-β1 regulates the proliferative potential of ER+ cells during mouse mammary development. To our knowledge this is the first mouse model to demonstrate a mechanism controlling the size of the ER+ subpopulation and a means of investigating how the dysregulation of this population contributes to the risk of developing breast cancer (see attached Abstract).

Over all this fellowship has supported research that has made important contributions to the scientific community providing a better understanding of the regulation of ER+ cells in mouse mammary gland during normal development. Mammary gland development in the mouse model to large extend replicates development in humans and therefore understanding this model provides information that can be extrapolated to the human breast development. The availability of this mouse model for studying the ER+ epithelial cell population will enable numerous studies to be done which will help in understanding the dysregulation that occurs in this cell population leading to abnormal growth in the mammary gland.

At personal level this DOD BRCP post-doctoral fellowship has provided an opportunity to transition from being a phytochemist to a breast cancer researcher. The final year of the fellowship spent in the Barcellos-Hoff laboratory has provided an opportunity and an enabling environment to learn about mammary gland biology focusing on various mouse models for studying normal

mammary gland development and carcinogenesis. Despite my very different training background in pharmacognosy/pharmaceutical chemistry, I am finally beginning to feel confident that a career in breast cancer research can be a reality for me.

LIST OF PERSONNEL THAT HAVE BEEN SUPPORTED BY THIS GRANT

Name and current position	Current institutional affiliation	Dates supported
Dr. Miaw-Sheue Tsai	E. O. Lawrence Berkeley National Lab.	Aug. 1999-July. 2000
Dr. Hellen Oketch-Rabah	E. O. Lawrence Berkeley National Lab.	Dec. 2001-Aug. 2003

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APPENDICES

Copy of papers and abstracts as follows

i. Tsai MS, Shamon-Taylor LA, Mehmi I, Tang CK, Lupu R. Oncogene. 2003 Feb 6;22(5):761-8.

ii. Tsai MS, Bogart DF, Castaneda JM, Li P, Lupu R. Oncogene. 2002 Nov 21;21(53):8178-85.

iii. Tsai MS, Bogart DF, Li P, Mehmi I, Lupu R. Oncogene. 2002 Jan 31;21(6):964-73

iv. Abstract No 134: Titled "Black Cohosh *(Cimicifuga racemosa)* does not have any estrogenic activity" (attached as Appendix I) for a poster presented at the International Scientific Conference on COMPLEMENTARY ALTERNATIVE & INTERGRATIVE Medicine Research that took place in Boston in April 12-14, 2002 at the Boston Marriot Copley Place, Boston MA

v. Abstract of the Black Cohosh (BC) paper that as been accepted for publication at the has been accepted for publication in the International Journal of Oncology.

vi. Abstract for a poster presented at a post doc day meeting at LBNL titled " Transforming Growth Factor-β1 Regulates the Proliferation of Estrogen Receptor Positive Mammary Epithelial Cells" Hellen A. Oketch-Rabah, Kenneth Ewan, G. Shyamala and Mary Helen Barcellos-HoffDepartment of Cell and Molecular Biology, Ernest Orlando Lawrence Berkeley National Laboratory , Berkeley, California 94720.

vii. Gordon Research Conference meeting. Mammary Gland Biology Meeting held at Roger Williams University RC meeting 06/01/2003 - 06/06/2003. Abstract Title "Transforming Growth Factor-β1 Regulates the Proliferation of Estrogen Receptor Positive Mammary Epithelial Cells *Hellen A. Oketch-Rabah, *Kenneth Ewan, Shraddha Ravani, *Shyamala G. and *Mary Helen Barcellos-Hoff.

viii. The modified SOW (SOW2) for year 2002/3: "The Role of TFG-β1 in the Regulation of Estrogen Receptor During Mouse Mammary Development and Carcinogenesis"

ix Tsai MS, Hornby AE, Lakins J and Lupu, R. Cancer Research 2000 Oct 60(20)5603-7

Appendix I

Oncogene (2003) 22, 761–768

www.nature.com/onc

SHORT REPORT

Blockage of heregulin expression inhibits tumorigenicity and metastasis of breast cancer

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The growth factor heregulin (HRG), expressed in about 30% of breast cancer tumors, activates the *erb*B-2 receptor via induction of heterodimeric complexes of *erb*B-2 with *erb*B-3 or *erb*B-4. HRG induces tumorigenicity and metastasis of breast cancer cells. Our investigation into whether HRG is a factor likely to promote tumor formation independently of *erb*B-2 overexpression concludes that blockage of HRG expression suppresses the aggressive phenotype of MDA-MB-231 breast cancer cells by inhibiting cell proliferation, preventing anchorage-independent growth, and suppressing the invasive potential of the cells *in vitro*. More importantly, we observed a marked reduction in tumor formation, tumor size, and a lack of metastasis *in vivo*. These studies were achieved by blocking HRG expression in MDA-MB-231 cells using an HRG antisense cDNA. In the search for the mechanism by which blockage of HRG reverts this aggressive phenotype, we discovered that the cells in which HRG is blocked exhibit a marked decrease in *erb*B activation and a significant reduction in MMP-9 activity, demonstrating a direct causal role in HRG induction of tumorigenicity. Our study is the first report and serves as a proof of the concept that HRG is a key promoter of breast cancer tumorigenicity and metastasis independently of *erb*B-2 overexpression and should be deemed a potential target in developing therapies for breast cancer.
Oncogene (2003) **22,** 761–768. doi:10.1038/sj.onc.1206130

Keywords: HRG; antisense; tumorigenicity; metastasis; breast cancer

Growth factors and their receptors have been implicated in playing an important role in the development and progression of cancer. It is known that signaling from the epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors (HER1/EGFR, HER2/*erb*B-2, HER3/*erb*B-3, and HER4/*erb*B-4) is involved in growth regulation of breast cancer cells (Stern, 2000). The role of EGFR in certain types of cancer is well established. Amplification of the *erb*B-2 gene is found in 20–30% of breast cancer patients and correlates with a poor prognosis (Slamon *et al.*, 1987), but the clinical relevance of *erb*B-3 and *erb*B-4 has yet to be determined. Unlike other members of the EGFR family, the ligand for *erb*B-2 has not yet been identified. However, *erb*B-2 can be activated by its own overexpression and homodimerization (Pierce *et al.*, 1991), or can be transactivated by heregulin (HRG) (Lupu *et al.*, 1992a, b). The HRG/*neu* differentiating factor (NDF) family of polypeptide growth factors binds either to *erb*B-3 or *erb*B-4 receptors and indirectly induces activation of *erb*B-2 through the formation of *erb*B-2: *erb*B-3 or *erb*B-2: *erb*B-4 heterodimers (Lupu *et al.*, 1990; Plowman *et al.*, 1993; Sliwkowski *et al.*, 1994). It is believed that the effects of HRG are mediated primarily through *erb*B-2, because functional blocking of *erb*B-2 inhibits HRG-induced cellular proliferation and transformation (Alimandi *et al.*, 1995). Numerous studies from others and from our laboratory have shown that biological response to HRG seems to depend directly upon the level of *erb*B-2 expression in breast cancer cells. In cells that overexpress *erb*B-2, low concentrations of HRG exhibit mitogenic stimulation, whereas high levels of HRG or constitutive expression of HRG induce growth arrest, cellular differentiation, or apoptosis (Lupu *et al.*, 1992a; Bacus *et al.*, 1992; Guerra-Vladusic *et al.*, 1999; Tripathy and Benz, 1992). In contrast, HRG at all concentrations stimulates proliferation in breast cancer cells that express low levels of the *erb*B-2 receptor (Lupu *et al.*, 1995).

Although overexpression of *erb*B-2 is a marker of poor prognosis in breast cancer, 70% of breast cancers overexpressing *erb*B-2 are characterized as noninvasive intraductal carcinoma (Paik *et al.*, 1990). This indicates that *erb*B-2 alone may not be sufficient for developing metastatic phenotypes and may require additional regulators for tumor progression. Interestingly, our data have shown that HRG is overexpressed in nearly 30% of breast cancer tumor biopsies that do not overexpress *erb*B-2 (Cardillo *et al.*, 1995). We have further shown that HRG expression in breast cancer cell

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lines and in breast tumor biopsies is inversely correlated with overexpression of *erb*B-2 and expression of estrogen receptor (ER) (Cardillo *et al.*, 1995). Our previous data demonstrate that HRG promotes tumorigenicity and metastasis of breast cancer cells that do not overexpress any of the *erb*B receptors (Tang *et al.*, 1996).

Significantly, the population of breast tumors that overexpresses HRG is distinct from the population overexpressing *erb*B-2, (Cardillo *et al.*, 1995). One such *in vitro* system manifesting this clinical observation is the ER-negative MDA-MB-231 human breast cancer cell line. These cells overexpress HRG, and are highly invasive *in vitro*, and tumorigenic and metastatic *in vivo*. This cell line expresses low levels of *erb*B-2 and *erb*B-3, (Cardillo *et al.*, 1995). We have further shown that antibodies generated against HRG markedly reduce *in vitro* growth, motility, and invasion of breast cancer cells that overexpress HRG, indicating that HRG is essential for breast cancer cell proliferation, motility, and invasion *in vitro* (Hijazi *et al.*, 2000). In the light of these observations, we hypothesize that HRG is a tumor-promoting factor that acts independently of *erb*B-2 overexpression. In this scenario, blockage of HRG expression will result in cell growth inhibition, reduction of tumorigenicity, and suppression of metastasis. We hypothesize that these events will occur in cells (or tumors) that express low levels of *erb*B-2 and high levels of HRG, such as the MDA-MB-231 breast cancer cells. It is important to look at other extremely aggressive tumors that do not overexpress *erb*B-2 and that require other biological therapies. Results from our present study strongly provide the proof of the concept that it should be plausible to target HRG for a large population of breast cancer patients (about 30%), those whose tumors overexpress HRG and express low levels of *erb*B-2.

To block HRG expression, a eukaryotic expression vector (pRC/CMV) was constructed with the HRG-β2 cDNA (amino acids 1–426) oriented from 3′ to 5′ end, that is, in an antisense direction, and subsequently transfected into MDA-MB-231 cells. Several HRG antisense (HRG/AS) clones were isolated and the presence of antisense HRG mRNA was confirmed by the RNAse protection assay (data not shown). Also generated were multiple clones of vector-transfected MDA-MB-231 (231/V) cells, all of which behaved similarly to the wild-type cells. Two MDA-MB-231 HRG/AS clones (C6 and C31) and one vector clone (231/V) are characterized further. The HRG protein expression was determined by Western blot analysis using an anti-HRG rabbit polyclonal antibody generated in our laboratory (Tang *et al.*, 1996). Conditioned media from C6, C31, and 231/V were collected and the HRG protein was purified by heparin chromatography as previously described (Lupu *et al.*, 1992b). Expression of the 45 kDa HRG protein was significantly reduced by 25–30-fold in the C6 cells and was low to undetectable in the C31 cells, as compared to the 231/V cells (Figure 1a). The biological activity of the remaining HRG expressed in clones C6 and C31 was next examined (as previously described – Guerra-Vladusic *et al.*, 1999) by HRG's ability to induce p185 tyrosine phosphorylation in MDA-MB-453 cells, which overexpress *erb*B-2 and express low levels of *erb*B-3 and *erb*B-4. Our results demonstrate that the ability of the remaining HRG in the HRG/AS cells to induce p185 tyrosine phosphorylation was extremely low to nearly undetectable, comparable to that in untreated cells (Figure 1b). In contrast, the 231/V cells induced *erb*B receptor tyrosine phosphorylation to an extent similar to cells treated with exogenous HRGβ1 (Figure 1b). As expected, the decreased level of HRG expression in the HRG/AS clones correlates with the inability of their conditioned media to induce *erb*B activation in MDA-MB-453 cells. These results demonstrate that the expression of HRG/AS DNA specifically and effectively blocks translation of the HRG mRNA into protein, and therefore

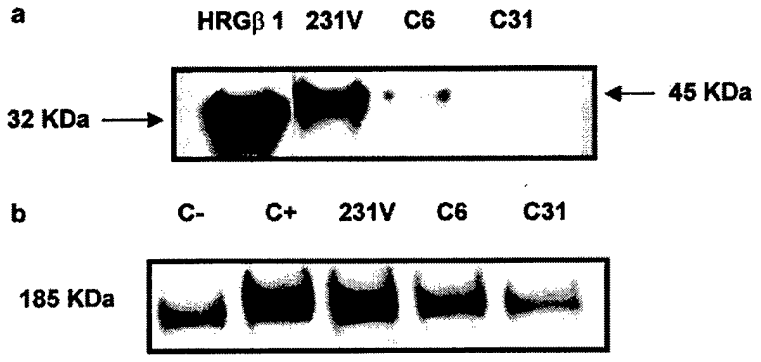

Figure 1 HRG expression and its ability to induce *erb*B-2 tyrosine phosphorylation are diminished in the HRG-AS clones. (**a**) Partially purified HRG from the conditioned media (CM) collected from vector-transfected cells (231/V) and HRG/AS-transfected cells (C6 and C31) was detected by Western blot analysis with an antibody against HRG that recognizes a 45-kDa protein (Tang *et al.*, 1996; Guerra-Vladusic *et al.*, 1999). Recombinant HRG-β1 protein (32 kDa) was used as a positive control. (**b**) Induction of p185 tyrosine phosphorylation of the *erb*B receptors in MDA-MB-453 cells. Partially purified HRG derived from the CM collected from 231/V, C6, and C31 cells (**a**) was used to determine p185 tyrosine phosphorylation by Western blot analysis using an anti-phosphotyrosine antibody (Tang *et al.*, 1996). MDA-MB-453 cells were treated in the presence or absence of HRG-β1 protein, denoted as positive (C+) and negative (C−) controls, respectively

significantly reduces HRG expression and its biological activity.

MDA-MB-231 is one of the most aggressive breast cancer cell lines, and these cells grow rapidly *in vitro*. Thus, the effect of blocking HRG expression on the anchorage-dependent growth of the cells was examined first. As shown in Figure 2a, the proliferation rate of HRG/AS clones C6 and C31 was decreased 35–50%, as compared to the 231/V cells. It is also known that HRG-overexpressing cells, such as MDA-MB-231, grow in an anchorage-independent manner. To determine whether inhibition of HRG expression had any effect on the ability of cells to grow as anchorage-independent cells, the HRG/AS and 231/V cells were tested in the soft agar assay as previously described (Tang *et al.*, 1996). Colonies with size between 60 and 100 μm were quantified using a soft agar colony counter. As shown in Figure 2b, although the total number of colonies of the C6 cells was not significantly different from that of the 231/V cells, the C6 colonies were generally at the smaller end of the size range (about 60 μm). On the other hand, the C31 cells showed a 50% reduction in anchorage-independent growth, as compared to the 231/V cells. Therefore, the decrease in HRG expression in MDA-MB-231 cells not only inhibits the anchorage-dependent growth, but also decreases the ability of the cells to grow in anchorage-independent manner.

MDA-MB-231 cells display stellar-like growth patterns in the Matrigel outgrowth assay (Sommers *et al.*, 1994). We have previously shown that an HRG-neutralizing antibody prevents MDA-MB-231 cells from developing stellar-like patterns in these assays (Hijazi *et al.*, 2000). Thus, we predicted that the HRG/AS clones would no longer reveal stellar-like patterns as seen in MDA-MB-231 cells. In this study, cells were plated on a Matrigel layer and grown for 7 days as previously described (Hijazi *et al.*, 2000). Neither of the HRG/AS clones was able to grow with stellar-like patterns as compared with the 231/V cells (Figure 2c). The C6 cells formed small foci, and migrated through the surrounding matrix. The C31 cells were not able to form proliferative foci, nor did they migrate through the Matrigel. In contrast, the 231/V cells formed large foci with stellar-like patterns, moving outward across the Matrigel as they replicated, and forming wide pathways of multiple cells through the surrounding matrix extensively (Figure 2c). These results imply that HRG is necessary for breast cancer growth and invasiveness. We then tested the ability of the HRG/AS cells to migrate and invade using the Boyden chamber assay as previously described (Hijazi *et al.*, 2000; Tsai *et al.*, 2000) Chemomigration is tested using a collagen matrix, and chemoinvasion is assessed with a Matrigel matrix. Both chemomigration and chemoinvasion of the C6 cells was reduced by 50% as compared with the 231/V cells; in the C31 cells, chemoinvasion was reduced by 75% and chemomigration was completely abolished, when compared with the vector cells (Figure 2d). As expected, the 231/V cells migrated and invaded rapidly through both the collagen and Matrigel matrices (Figure 2d).

It is noteworthy that HRG protein expression is clearly decreased in clone C6, but not completely blocked as in clone C31 (Figure 1a). The threshold expression of HRG in clone C6 appears sufficient to induce *erb*B-activation (Figure 1b). Moreover, the effect of HRG threshold expression is more evident for observed intermediate levels of anchorage-independent growth in clone C6 (Figure 2b), in which a marked decrease in colony size was more evident than a decrease in colony number, as compared with 231 V cells. Furthermore, the C6 cells displayed moderate reduction in their outgrowth ability in Matrigel, in chemomigration, and in emoinvasion, as compared with the 231 V and C31 cells (Figure 2c, d). All of these data indicate that a steady-state decrease in the aggressiveness of MDA-MB-231 cells depends directly upon the threshold level of HRG. The data presented to this point, clearly demonstrate that blockage of HRG expression reduces cellular proliferation, inhibits anchorage-independence, blocks Matrigel outgrowth, and decreases chemoinvasive and chemomigration behavior. All together, these data demonstrate that HRG is necessary to induce aggressive phenotypes of the MDA-MB-231 cells.

To assess the effect that blockage of HRG has *in vivo*, the HRG/AS cells were inoculated into the mammary fat pads of 3–4-week-old athymic nude mice. At 4 weeks after cell inoculation, we observed that the C6 and C31 cells showed a significant decrease in tumor size and in total weight, as compared with the 231/V cells, which develop largely vascularized tumors similar to the human invasive breast carcinomas in athymic nude mice (Figure 3). The variability in tumor size among each group was not significant. However, the HRG/AS-derived tumors were extremely small (Figure 3) and did not appear vascularized (data not shown). The mice containing the 231/V-derived tumors were killed 4 weeks after cell inoculation, since the tumors reached the largest size allowable. Mice that contained the HRG/AS-derived tumors were kept for an additional 8 weeks, during which time they did not show a significant change in tumor intake and tumor size. It is known that MDA-MB-231 cells are not only tumorigenic but also metastatic. As expected, the 231/V cells that developed into large tumors were readily metastatic, and the metastatic foci appeared in the liver and the lung (Figure 3b). In contrast, neither the C6- nor the C31-derived tumors metastasized even 12 weeks after the initial cell inoculation. Our *in vivo* results are in agreement with the *in vitro* data. The data presented here without a doubt demonstrates that HRG controls the tumorigenic and metastatic potential of many breast cancer cells in vivo. It appears that blockage of HRG blocks the signaling machinery necessary for these cells to grow, invade and metastasize.

Our studies lead us to conclude that HRG is a tumor-promoting factor, expression of which is critical for the progression of breast carcinomas. The data presented here support the notion that the behavior of breast cancer cells in culture correlates to varying levels of HRG expression (Hijazi *et al.*, 2000). It appears that the different threshold levels of HRG expression in clones

C6 and C31 promote slightly different *in vitro* phenotypes (Figures 1 and 2). Although there is a minor difference in the threshold HRG expression in each HRG/AS clone, the *in vivo* behavior of all the HRG/AS cells is similar (Figure 3). Our results are of great importance for the development of therapies that will be

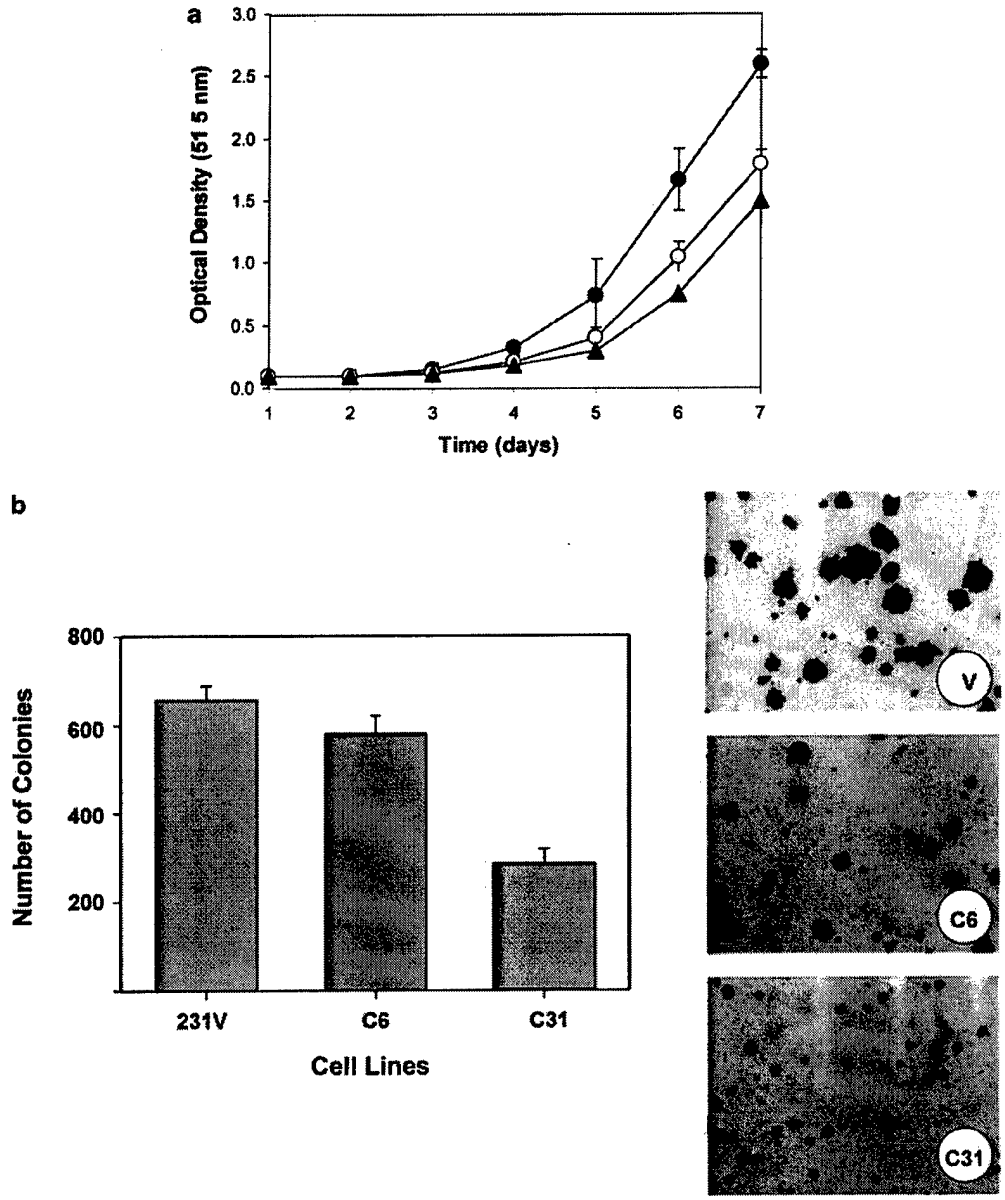

Figure 2 Obstruction of HRG expression results in a significant decline in the aggressiveness of MDA-MB-231 *in vitro*. (**a**) Anchorage-dependent growth was decreased in HRG/AS cells. Cells (1000/well), 231/V (•), C6 (○), and C31 (▲), were seeded in 96-well plates. Growth was evaluated over a period of 7 days by fixing cells with trichloroacetic acid, staining with sulforhodamine B, and measuring optical density at 515 nm. (**b**) HRG/AS cells were significantly less clonogenic than the 231/V cells in soft agar. Cells (5000/well) were plated in triplicate in agar layer and grew for 2 weeks at 37°C. Colonies of 60–100 μm were stained with *p*-iodonitrotetrazolium purple and quantified with an AccuCount 2000 automatic colony counter (Grunt *et al.*, 1995). (**c**) The pattern of Matrigel outgrowth of the HRG/AS clones was markedly changed as compared with the 231/V cells, from a stellar-like pattern with invasive components in the 231/V to small foci with limited invasive components, if any, in the HRG/AS cells. Cells (25 000/well) were plated in triplicate in Matrigel in a 12-well plate, and microphotographs were taken at day 7 as previously described (Hijazi *et al.*, 2000). (**d**) The HRG/AS showed decreased chemoinvasion and chemomigration activities. Boyden chamber assay of HRG/AS clones was performed as previously described (Tsai, *et al.*, 2000; Hijazi *et al.*, 2000). In brief, cells (20 000 cells/well) were plated in quadruplicate in the upper chamber of a 48-well Boyden chamber onto polycarbonate filters coated with either collagen IV or Matrigel in serum-free media. CM from NIH3T3 fibroblast was used as a chemoattractant in the lower chambers. After incubation for 6 h at 37°C, cells on the top surface were removed, and filters were then fixed, and stained with crystal violet. The number of cells that migrated through the pores was assessed by microscopy. Similar results were obtained from at least four to five independent experiments

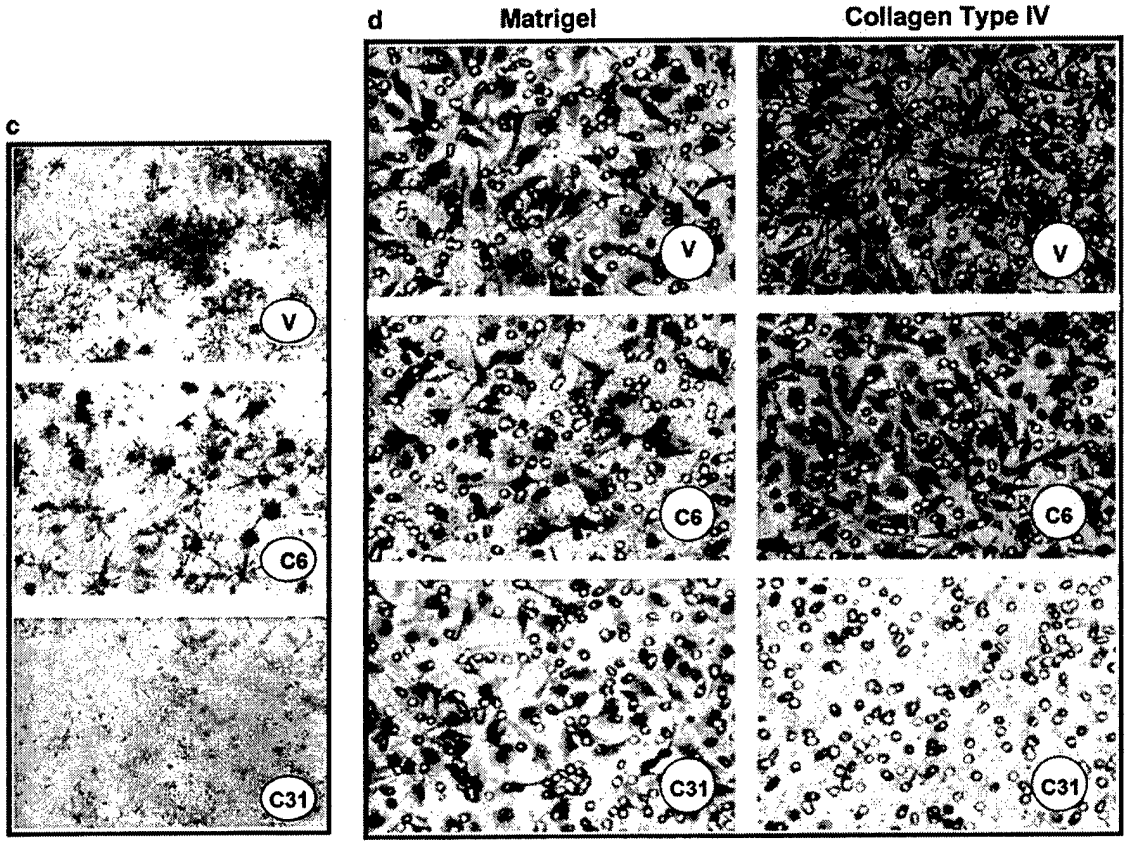

Figure 2 *Continued*

aimed at targeting HRG for use in those breast cancer patients in whom increased expression of HRG is observed. We do postulate that those tumors are likely to develop tumor metastasis. Our initial studies demonstrate that 30% of invasive breast carcinomas express high levels of HRG and its expression is correlated with aggressiveness of the tumor, lack of ER and low expression of erbB-2 (data not shown).

To understand the mechanism by which blockage of HRG expression reverts the aggressive phenotype of MDA-MB-231 cells, we examined whether HRG-mediated signaling pathways are altered in the HRG/AS cells. Expression of the *erb*B-2 and *erb*B-3 receptors was assessed by performing immunoprecipitation under nonreducing conditions, followed by immunoblotting for *erb*B-2 and/or *erb*B-3 receptors using specific receptor antibodies. To evaluate the level of *erb*B-2 and *erb*B-3 tyrosine phosphorylation, immunoprecipitations as described above were followed by immunoblotting for phosphotyrosine using an antiphosphotyrosine antibody. We demonstrate that the level of the *erb*B-2 protein, although low, was unchanged in the 231/V and HRG/AS cells (Figure 4a, top panel). In contrast, the level of *erb*B-2 autophosphorylation was decreased in the C6 and C31 cells, as compared to the 231/V cells (Figure 4a, middle panel). The basal level of *erb*B-3 protein was low to undetectable (data not shown) in all of the cells, and the level of *erb*B-3 tyrosine phosphorylation was markedly decreased in the C6 and C31 cells in comparison with the 231/V cells (Figure 4a, bottom panel). Our data clearly suggest that by blocking HRG expression, a cascade of events leads to a decrease in both *erb*B-2 and *erb*B-3 tyrosine phosphorylation. Tyrosine phosphorylation of *erb*B receptors is one of the signaling events that modulate cell proliferation, tumor formation, and metastatic behavior of breast cancer cells. This is an important observation supported by the previous data that the primary *erb*B heterodimer in human breast carcinomas is *erb*B-2: *erb*B-3, which is correlated with an aggressive phenotype (Chen *et al.*, 1996). MDA-MB-231 cells do not overexpress *erb*B-2 and *erb*B-3. From our data, it is clear that the growth and the receptor signaling events in these cells are not dependent upon *erb*B-2 overexpression, but rather on ligand (HRG) induced receptor heterodimerization. Thus, obstruction of HRG expression in MDA-MB-231 cells disrupts HRG-induced heterodimerization between erbB-2 and erbB-3, leading the lower erbB-2 activation. Interestingly, MDA-MB-231 cells express high levels of EGFR (*erb*B-1) and secrete TGFO and EGF, both of which directly activate and phosphorylate EGFR in an autocrine manner. Aggressiveness of

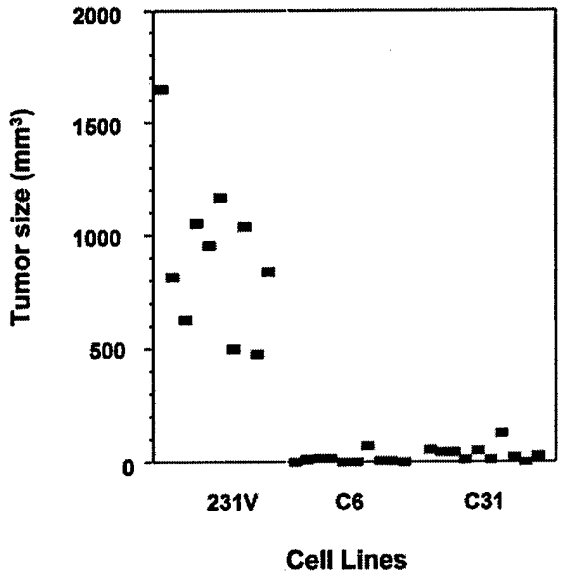

Figure 3 Blockage of HRG expression suppresses tumorigenicity and metastasis of MDA-MB-231 cells *in vivo*. (**a**) Athymic nude mice (3–4-week old) were implanted with 231/V, C6 and C31 cells (5×10^5/site) into the mammary fat pads as described previously (Tang *et al.*, 1996). Tumor size was calculated by three-dimensional measurements. The tumors produced by the 231/V cells ranged from 500 to 1600 mm³, and no significant tumor formation was seen in mice inoculated with the HRG/AS cells (C6 and C31). Studies were performed for 12 weeks; however, all the measurements were performed 4 weeks after inoculation. No significant changes in tumor development were observed in the HRG/AS-inoculated mice. Control 231/V mice were sacrificed after 4 weeks because of the large appearance of the tumors. (**b**) Metastases derived from the primary tumors were observed by H&E staining in the sections of liver (a) and lungs (b) from mice inoculated with the 231/V cells. No metastases were observed in the HRG/AS-inoculated mice. Arrows indicate the detection of human breast cancer epithelial cells

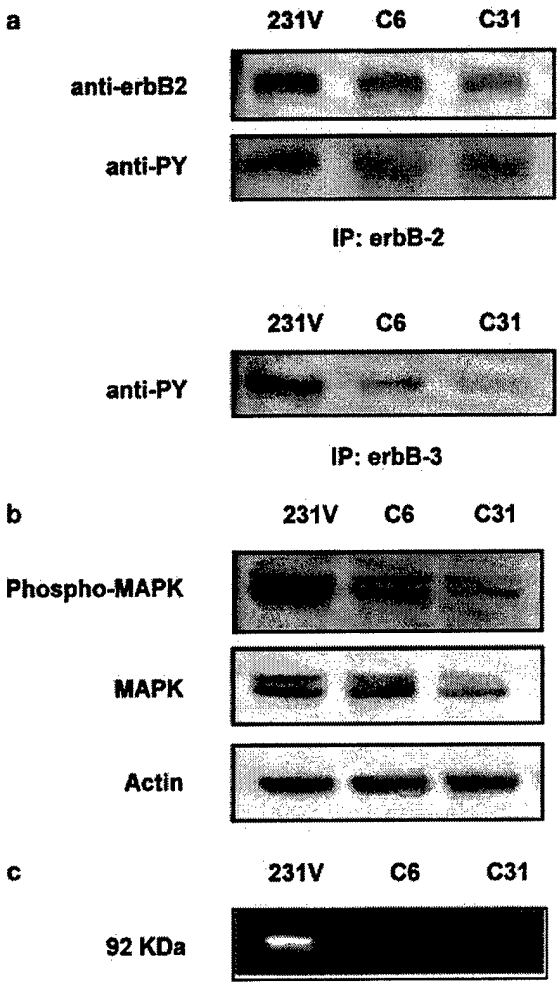

Figure 4 Inhibition of HRG expression causes decreased activation of *erb*B-2, MAPK, and MMP-9 activity in MDA-MB-231 cells. (**a**) Expression and activation of *erb*B receptors were downregulated in HRG/AS cells. The *erb*B receptors were immunoprecipitated from lysates prepared from 231/V, C6, and C31 cells, and immunoblotted for *erb*B-2 (top panel), *erb*B-2 tyrosine phosphorylation (middle panel), and *erb*B-3 tyrosine phosphorylation (bottom panel). (**b**) Activation of MAPK was decreased in HRG/AS clones. Cells (231/V, C6, and C31) were serum-starved for 24 h. Cell lysates were collected, separated on SDS–PAGE, and immunoblotted with antibodies against phosphorylated MAPK (top panel), MAPK (middle panel), and actin (bottom panel). (**c**) MMP-9 enzymatic activity was diminished in HRG/AS cells. Conditioned media were collected and concentrated 100 × from the 231/V, C6, and C31 cells after 72-h serum starvation. Equal amounts of protein were loaded onto an SDS–gelatin–PAGE and analysed for MMP-9 activity using reverse zymography as described previously (Hua and Muschel, 1996)

MDA-MB-231 cells, however, is not dependent upon EGFR activation. It has been shown that blockage of EGFR/EGF disruption does not lead to reduction of tumor formation in mDA-MB-231 cells, although these cells overexpress EGFR. Namely, EGFR does appear to be a relevant pathway for tumorigenic phenotype of these cells. Furthermore, inhibition of HRG has no effect on EGFR activation (data not shown). This is an extremely important finding, because it is the first indication that blockage of HRG expression inhibits tumorigenicity and abolishes the metastatic processes of the cells by perhaps inhibiting a large cascade of events driven by the erbB-signaling pathway.

Next, we examined MAPK, a downstream effector molecule of the *erb*B receptor tyrosine phosphorylation. It has been shown that activation of *erb*B-2 by HRG leads to breast cancer proliferation, presumably by inducing activation of the MAPK and PI3 K (phosphotidylinositol-3 kinase)/Akt pathways (Sepp-Lorenzino *et al.*, 1996; Reese and Slamon, 1997; Lim *et al.*, 2000). We then postulated that inhibition of HRG expression would have an impact on the erbB-downstream signaling molecules *Ras/MAPK* which may be in part, controlling cell growth and proliferation. We found that phosphorylated MAPK (ppMAPK) was greatly decreased in the C6 and C31 cells by a more than 80% reduction, as compared with the 231/V cells cultured under the same conditions (Figure 4b, top panel). To demonstrate that a decrease in ppMAPK was specific, and not because of a decrease in the total MAPK protein, the levels of the MAPK protein were also assessed. The total MAPK protein levels in the C6 and

C31 cells were not altered as compared to 231/V cells (Figure 4b, middle panel). Protein loading control was shown by using an anti-actin antibody to ensure that equal amounts of protein were analysed (Figure 4b, bottom panel).

Through this, we demonstrate that HRG-induced tumorigenicity and invasiveness is, at least in part, regulated through *erb*B receptor-mediated *Ras*-dependent MAPK pathways. These events have previously been shown *in vitro* and in other model systems, which have demonstrated that HRG promotes cellular proliferation through the *Ras*-dependent MAPK (Sepp-Lorenzino *et al.*, 1996). It has also been shown that upon activation by HRG, *erb*B-2 becomes phosphorylated and bound to the SH2 domain of the Grb2 (Lim *et al.*, 2000), which in turn leads to activation of MEK and MAPK (Reese and Slamon, 1997). It should be noted that MDA-MB-231 cells express activated Ras. However, it is most likely that nonactivated Ras exists as *a* predominant form (as in most cells), which may be activated through the HRG/*erb*B signaling pathway. Blockage of HRG expression promotes a decrease in *erb*B-3 and *erb*B-2 activation as well as a decrease in activation of the downstream signaling molecules. This is the first report demonstrating that, in fact, HRG is *a* key regulator of these events in breast carcinomas, and that blockage of HRG expression leads to a phenotypic regression, from a very aggressive and metastatic phenotype to a nonaggressive and nonmetastatic phenotype. These changes are solely mediated by the blockage of HRG expression and the impact that this blockage has on erbB-activation.

Matrix metalloproteases (MMPs) have been associated with tumor cell invasion and metastasis (Curran and Murray, 1999). Since we had initially seen (Atlas *et al.*, 2003), that HRG induced MMP-9 activity, we postulated that one of the mechanisms by which blockage of HRG caused inhibition of metastasis was perhaps by blockage of an extracellular matrix degrading enzyme, such as the MMP-9. We have also shown that a specific MMP-9 inhibitor blocks the invasive phenotype of HRG-expressing cells, but not cells that do not express HRG (Liu *et al.*, 1999; Atlas *et al.*, 2003). Thus, we investigated MMP-9 expression and activity in the antisense expressing cells. We demonstrated the MMP-9 expression was not changed between the control and the antisense HRG cells, but a tremendous decrease in MMP-9 activitiy. Assessment of MMP activity was performed using a reverse zymography as previously described (Hua and Muschel, 1996; Lee *et al.*, 1996). A striking difference in the MMP-9 activity was found. MMP-9 was low or undetectable in the C6 and C31 cells, as compared with 231/V cells, which secreted high levels of MMP-9 enzymatic activity (Figure 4c). MMP-9 is a metalloprotease that plays a role in the degradation of type IV collagen (gelatin), and has been found highly expressed in breast carcinomas (Himelstein *et al.*, 1997). Our results strongly suggest that the enzymatic activity of MMP-9 is associated with HRG expression, and that both are involved in the invasive and metastatic phenotype of MDA-MB-231 cells. Moreover, our results are consistent with previous observations, in which increased production of pro-MMP-9 and secretion of MMP-9 are associated with metastasis induced by activated *Ras*-transformed breast cancer cells (Tsang and Crowe, 2001). MMP-9 is required for this process, because a ribozyme directed against MMP-9 abolishes the ability of the cells to metastasize (Hua and Muschel, 1996). Moreover, it has been shown recently that MMP-9 expression is activated by HRG (Yao *et al.*, 2001), and that the *erb*B-2-mediated *Ras*-dependent MAPK pathway is involved in the upregulation of MMPs (Tsang and Crowe, 2001). We have previously demonstrated that HRG promotes tumorigenicity in part via upregulation of an angiogenic factor Cyr61 (Tsai *et al.*, 2000), and also via receptor tyrosine phosphorylation and MAPK activation (Atlas *et al.*, 2003; Tsai *et al.*, 2002). However, this is the first report to demonstrate the importance of MMP-9 activity driven by HRG expression, as well as the function of MMP-9 in promoting metastasis (Atlas *et al.*, 2003).

In summary, our present study demonstrates indisputably the crucial role that HRG plays in acquiring an aggressive phenotype of the human breast cancer cells. By effective blockage of HRG expression using stable transfection with an antisense RNA expression vector for HRG, we show nearly complete reversion of the tumorigenic and metastatic phenotype of the MDA-MB-231 cells. These results are of critical significance, endorsing the view that the aggressive phenotypes are not dependent on *erb*B-2 overexpression, but on expression of HRG and erbB receptor activation in several breast cancer cells, as manifested clinically in certain populations of human breast carcinomas. Examples include MDA-MB-231 presented here, and two other breast cancer cell lines HS578 T and BT549, in which the data were very similar (data not shown). Moreover, our results provide evidence into the possible mechanism of blocking HRG action in halting breast cancer progression through inactivation of HRG-induced *erb*B signaling and the subsequent decrease in activated MAPK, MMP-9 activity and possibly AKT activation.

Even though the two distinct breast cancer populations – one overexpressing *erb*B-2 with low levels of HRG, and the other expressing HRG but not *erb*B-2 – may appear to employ the same signaling pathways in growth control, effective treatments of breast carcinomas should be specifically targeted. This is the first report addressing the possibility of targeting HRG in breast carcinomas that do not overexpress *erb*B-2, and demonstrating the critical role of such intervention against HRG. We show clearly here that blockage of HRG expression results in nearly complete inhibition of tumor formation and obstruction of metastasis *in vivo*. Our unique and novel findings furnish a proof of concept and provide new insights into the development of potential therapies targeted at blocking HRG expression and/or its action and thereby halting the progression of breast cancer. This will benefit a large breast cancer population whose tumors overexpress HRG, the 30% of patients who have invasive breast

carcinomas and for whom conventional therapies are known to fail.

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Appendix II

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Cyr61 promotes breast tumorigenesis and cancer progression

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Cyr61, a member of the CCN family of genes, is an angiogenic factor. We have shown that it is overexpressed in invasive and metastatic human breast cancer cells and tissues. Here, we investigated whether Cyr61 is necessary and/or sufficient to bypass the 'normal' estrogen (E2) requirements for breast cancer cell growth. Our results demonstrate that Cyr61 is sufficient to induce MCF-7 cells to grow in the absence of E2. Cyr61-transfected MCF-7 cells (MCF-7/Cyr61) became E2-independent but still E2-responsive. On the other hand, MCF-7 cells transfected with the vector DNA (MCF-7/V) remain E2-dependent. MCF-7/Cyr61 cells acquire an antiestrogen-resistant phenotype, one of the most common clinical occurrences during breast cancer progression. MCF-7/Cyr61 cells are anchorage-independent and capable of forming Matrigel outgrowth patterns in the absence of E2. ERα expression in MCF-7/Cyr61 cells is decreased although still functional. Moreover, MCF-7/Cyr61 cells are tumorigenic in ovariectomized athymic nude mice. The tumors resemble human invasive carcinomas with increased vascularization and overexpression of vascular endothelial growth factor (VEGF). Our results demonstrate that Cyr61 is a tumor-promoting factor and a key regulator of breast cancer progression. This study provides evidence that Cyr61 is sufficient to induce E2-independence and antiestrogen-resistance, and to promote invasiveness *in vitro*, and to induce tumorigenesis *in vivo*, all of which are characteristics of an aggressive breast cancer phenotype.
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About 60% of human breast carcinomas expressing estrogen receptor (ER), are dependent upon estrogen (E2) for growth, and thus respond to treatment with an ER antagonist, tamoxifen (Tam). Many breast carcinomas, however, become less sensitive over time to estrogen, and thus more resistant to the endocrine treatment, developing into more aggressive tumors. Aggressiveness of breast cancer cells is commonly attributed to the ability of the cells to overcome E2 requirements for growth, and in most cases to acquire antiestrogen-resistance. However, the mechanism by which breast cancer progresses from an E2-dependent and antiestrogen-responsive phenotype to an E2-independent and antiestrogen-resistant phenotype has not yet been determined.

We have shown previously that expression of heregulin (HRG), a growth factor that activates the *erbB*-2/3/4 receptor signaling pathways, is closely associated with an invasive breast cancer phenotype (Cardillo *et al.*, 1995). We have further demonstrated that HRG induces breast cancer progression, as determined by the loss of ER function and E2 response, tumorigenicity, invasion, and metastasis (Tang *et al.*, 1996; Lupu *et al.*, 1995, 1996; manuscript submitted for publication). We have hypothesized that HRG induces activation of the *erbB* signaling pathways, leading to regulation of downstream genes that control cancer cell growth and tumor progression. We thus isolated and identified Cyr61, an angiogenic factor, which is differentially expressed in ER-negative, HRG-positive, invasive, and metastatic breast cancer cells, and in 30% of breast tumor biopsies (Tsai *et al.*, 2000). We showed that Cyr61 is important for HRG-mediated chemomigration and invasiveness of breast cancer cells *in vitro* (Tsai *et al.*, 2000).

Cyr61 belongs to the CCN family of angiogenic regulators, which consists of Cyr61, CTFG, Nov, WISP-1, WISP-2, and WISP-3 (Lau and Lam, 1999). Cyr61 is a cysteine-rich, heparin-binding protein that is secreted and associated with the cell surface and the extracellular matrix (ECM) (Yang and Lau, 1991; Kireeva *et al.*, 1997). It has been shown that Cyr61 binds to integrins, such as $\alpha v\beta 3$, $\alpha IIb\beta 3$ and $\alpha 6\beta 1$ (Kireeva *et al.*, 1996a; Jedsadayanmata *et al.*, 1999; Chen *et al.*, 2000). Cyr61 mediates cell adhesion, stimulates cellular migration, enhances growth factor-induced DNA synthesis in fibroblasts and endothelial cells, and increases chondrogenesis in mesenchymal cells (O'Brien and Lau, 1992; Kireeva *et al.*, 1996b,

1997; Frazier *et al.*, 1996; Kolesnikova and Lau, 1998). Moreover, Cyr61 stimulates an integrin $\alpha v\beta 3$-dependent chemotaxis of endothelial cells (Babic *et al.*, 1998). Most significantly, expression of Cyr61 enhances neovascularization and tumor formation of human tumor cells in immunodeficient mice (Babic *et al.*, 1998, 1999; Xie *et al.*, 2001).

To determine whether expression of Cyr61 is necessary and/or sufficient to promote breast cancer progression, Cyr61 was introduced into the MCF-7 breast cancer cells, which are ER-positive, E2-responsive *in vitro*, E2-dependent *in vivo*, and never metastasize *in vivo*. MCF-7 cells were stably transfected by electroporation with a eukaryotic expression vector, pcDNA3.1/zeocine(−) (Invitrogen), containing the full-length cDNA of the human Cyr61 gene (MCF-7/Cyr61), or with an empty vector (MCF-7/V) as a negative control. A number of Cyr61- (20 clones) and vector-transfected clones (10 clones) were isolated and characterized for the expression of Cyr61 at both the mRNA and protein levels. Cyr61 was highly expressed in the Cyr61-transfected cells as determined by the RNase protection assay (Figure 1a) and by Western blot analysis using conditioned media concentrated from the Cyr61 clones or vector control clones (Figure 1b). Importantly, the expression level of Cyr61 in MCF-7/Cyr61 was comparable to that in MDA-MB-231, which is an aggressive breast cancer cell line and naturally expresses high levels of Cyr61. Our results demonstrated a 10- to 35-fold increase in Cyr61 mRNA and/or protein in the MCF-7/Cyr61 cells, as compared with the wild type or MCF-7/V cells in which Cyr61 expression was very low or nearly undetectable (Figure 1). Since Cyr61 mRNA and protein expression did not vary significantly, and their cellular behavior was similar in most of the clones, we chose to present the data obtained in one vector clone (V2) and two representative Cyr61 clones (C6 and C20).

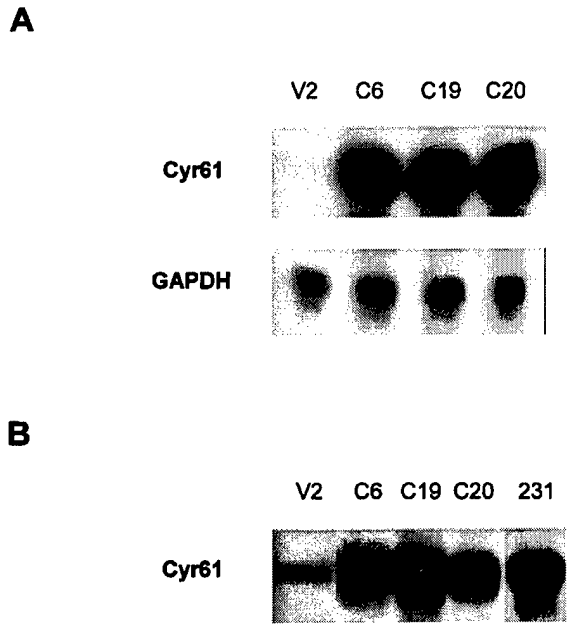

Figure 1 Expression of Cyr61 in MCF-7/Cyr61 clones. (**a**) MCF-7 cells were stably transfected by electroporation with a eukaryotic expression vector, pcDNA3.1/zeocine(−) (Invitrogen), containing the full-length cDNA of the human Cyr61 gene, or with an empty vector as a negative control. Transfected MCF-7 cells were selected in the presence of antibiotic zeocine (200 μg/ml) for 2 weeks. Individual vector transfectants (MCF-7/V, 10 clones) and Cyr61 transfectants (MCF-7/Cyr61, 20 clones) were isolated and grown. Total RNA was isolated from subconfluent MCF-7/V2 and MCF-7/Cyr61 cells, and 30 μg of RNA was analysed for Cyr61 mRNA expression by RNAse protection assay as previously described (Tsai *et al.*, 2000). The GAPDH probe was used as an internal control for RNA loading. Representative vector (V2) and Cyr61 clones (C6, C19, and C20) were shown. (**b**) Subconfluent breast cancer cell lines were cultured in serum-free media for 48 h. Conditioned media were collected, concentrated 100×, and analysed by Western blotting with a rabbit polyclonal anti-Cyr61 antibody as previously described (Tsai *et al.*, 2000)

The MCF-7/Cyr61 cells showed a growth advantage in E2-depleted media, having a 3–5-fold increase in growth as compared with the MCF-7/V cells (Figure 2a). The average doubling time in E2-free conditions for the MCF-7/Cyr61 cells is approximately 36 h, in contrast with 72 h for the MCF-7/V2 cells (data not shown). These results demonstrate that MCF-7/Cyr61 cells acquire an E2-independent phenotype. We thus postulate that overexpression of Cyr61 provides MCF-7 cells a growth advantage to bypass their 'normal' estrogenic requirement for cellular proliferation. As expected, E2 stimulated the growth of MCF-7/V2 cells (Figure 2a) because MCF-7 cells are E2-responsive (Pratt and Pollak, 1993). Although the MCF-7/Cyr61 cells acquire E2-independence, these cells are still responsive to E2 (Figure 2a), resembling one of the clinical phenotypes found in women suffering from breast cancer. We next tested the ability of antiestrogens to block the E2 induction of cell growth. Two distinct antiestrogens were used, Tamoxifen (Tam) and ICI 182,780 (ICI). Tam, a well-known antiestrogen, functions as an agonist and antagonist through both transcriptional activation domains (AF1 and AF2) of ER. ICI, a pure antiestrogen, acts solely as an antagonist through the AF1 domain (MacGregor and Jordan, 1998). When MCF-7/Cyr61 cells were treated with ICI (Figure 2a), and Tam (data not shown), both antiestrogens reduced only the growth induced by E2 in MCF-7/Cyr61 cells. They were unable to block the E2-independent growth of the MCF-7/Cyr61 cells, indicating that Cyr61 provides a true growth advantage that is not inhibited by antiestrogens. In other words, both antiestrogens inhibited the growth of the cells only to the basal level elevated by overexpression of Cyr61; they were not able to reduce cell growth to the same level observed in the wild type or MCF-7/V2 cells in the absence of E2 (Figure 2a). The fact that both antiestrogens had similar effects supports the hypothesis that the growth stimulation by E2 is indeed mediated directly through ER and most probably not mediated through other secondary mechanisms.

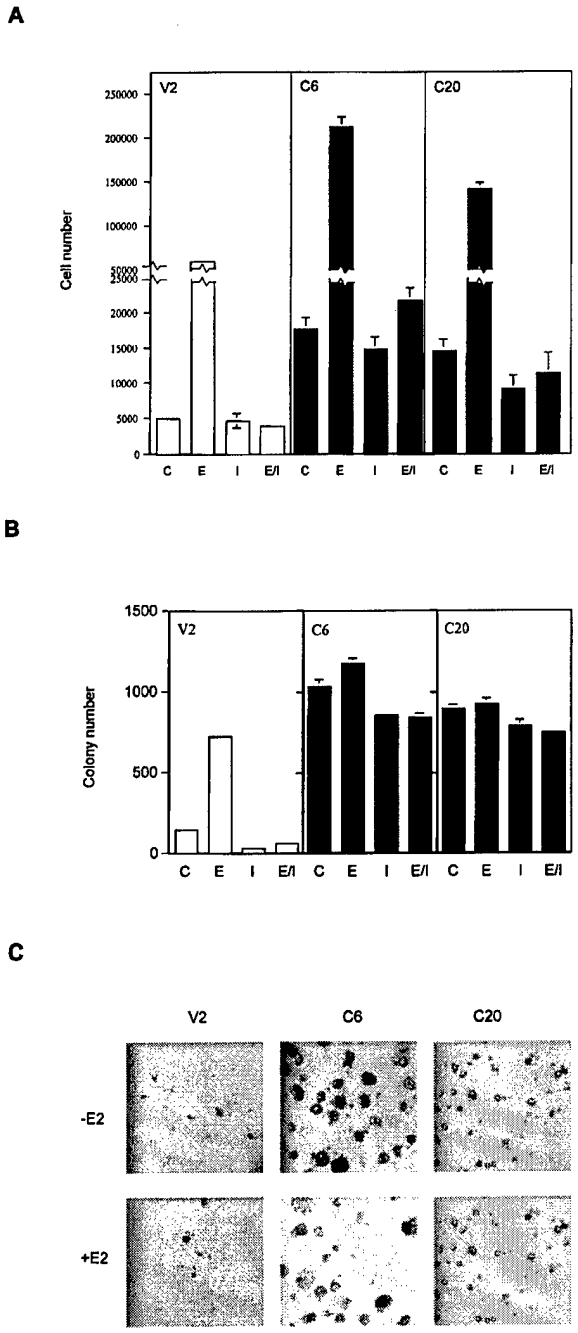

Figure 2 Induction of E2-independence and antiestrogen resistance of MCF-7 cells by Cyr61. (**a**) Subconfluent MCF-7/V2 and MCF-7/Cyr61 clones were cultured in media containing charcoal stripped fetal bovine serum (FBS) for 4 days and plated (2500 cells/well) in triplicate in 24-well plates for proliferation assays. Cells were incubated in the presence of solvent control (0.1% ethanol; C), E2 (1 nM; E), ICI (100 nM; I), and the combination of E2 and ICI (E/I). Cell number was counted at day 7. Data of two representative Cyr61 clones are shown from at least four independent experiments. Similar results were obtained from other Cyr61-overexpressing clones. (**b**) Subconfluent MCF-7/V2 and MCF-7/Cyr61 clones were cultured in E2-depleted media for 4 days and plated (20 000 cells/well) in triplicate in 6-well plates for anchorage-independent soft agar assay as previously described (Guerra-Vladusic *et al*., 1999). Cells were cultured in the presence of solvent control (C), E2 (E), ICI (I), and the combination of E2 and ICI (E/I) as described above for a. Data of two representative clones are shown from at least four independent experiments. Similar results were obtained from other Cyr61-overexpressing clones. (**c**) Subconfluent MCF-7/V2 and MCF-7/Cyr61 clones were cultured in E2-depleted media for 4 days and plated (5000 cells/well) in triplicate in 12-well plates in the presence or absence of E2 in the Matrigel Outgrowth assay as previously described (Hijazi *et al*., 2000). The outgrowth pattern was developed and photographed after a 10-day incubation. Two representative clones are shown with similar results from three independent experiments. Similar results were obtained from other Cyr61-overexpressing clones

To sum up, our data demonstrate that overexpression of Cyr61 stimulates cell growth of E2-dependent cells in the absence of E2, resulting in cells becoming E2-independent. On the other hand, E2 further enhanced cell proliferation of Cyr61-expressing cells, demonstrating that these cells, although independent of E2, are still responsive to E2. Consistent with our previous findings that E2 induces Cyr61 expression, and Tam and ICI blocks E2-induced Cyr61 expression (Tsai *et al*., 2002), here we demonstrated that both antiestrogens decrease E2-stimulated growth in Cyr61-expressing cells only to those levels already stimulated by overexpression of Cyr61. Therefore, it is overexpression of Cyr61 alone, which most probably accounts for the growth advantage observed in MCF-7 cells in E2-depleted conditions. Moreover, our *in vitro* studies represent a situation similar to what often occurs in human breast carcinomas *in vivo*, in which E2 can induce tumor growth (or not), in spite of levels of ER expression which are almost identical. Clinical trials have been conducted with E2 followed by chemotherapeutic drugs, based on the rationale that E2 stimulates tumor growth, allowing cells to enter the cell cycle, and thus provides a better environment for chemotherapeutic drugs to be more effective.

Cyr61-induced E2-independence became more evident when we further demonstrated that MCF-7/Cyr61 cells are anchorage-independent in the absence of E2 (Figure 2b), that is, these cells form colonies in soft agar assays. It is well established that MCF-7 cells are not anchorage-independent in the absence of E2. Colonies observed, if any, represent the background level for the colony formation assay. In general, the MCF-7/Cyr61 cells formed large colonies in the size range of 100–150 μm. E2 slightly stimulated the colony formation in the MCF-7/Cyr61 cells (Figure 2b). ICI was not able to block the colony formation induced by E2 in MCF-7/Cyr61 (Figure 2b). All other MCF-7/Cyr61 clones behaved similarly (data not shown). Neither vector nor wild type MCF-7 cells formed significant numbers of colonies in the absence of E2. Smaller colonies or single cells were seen in MCF-7/V2 cells in the presence of E2 (Figure 2b). As expected, E2 induced anchorage-independent growth of MCF-7/V cells, which was completely blocked by antiestrogens ICI (Figure 2b) and Tam (data not shown).

To determine whether Cyr61 promotes an invasive phenotype, MCF-7/Cyr61 cells were tested in the Matrigel outgrowth assay, which is frequently employed as a reliable system to assess *in vitro* invasiveness of breast cancer cells (Sommers *et al.*, 1994; Hijazi *et al.*, 2000). The MCF-7/Cyr61 clones C6 and C20 showed extensive outgrowth in Matrigel, the colonies appearing large and irregular in shape. In contrast, the MCF-7/V2 cells were not able to migrate through and proliferate in the Matrigel matrix, remaining as single cells in the matrix even in the presence of E2 (Figure 2c). Significantly, Cyr61 promotes outgrowth of MCF-7 cells in the Matrigel matrix in the absence of E2, suggesting that Cyr61 is capable of inducing a critical invasive phenotype of breast cancer cells in an E2-independent manner. These results strongly indicate that expression of Cyr61 enhances the *in vitro* invasiveness of breast cancer cells, which may thereby provide the appropriate milieu for these cells to migrate and perhaps metastasize *in vivo*. All other MCF-7/Cyr61 clones behaved similarly (data not shown).

It has been shown previously that a possible mechanism to acquire an E2-independent and anti-estrogen-resistant phenotype acts via the loss of ER expression and/or ER function. The ER expression was determined by RNase Protection assays; the ER function was determined as the ability of E2 to regulate the expression of E2-responsive genes (Govind and Thampan, 2001). We first examined the basal level of ER expression in MCF-7/Cyr61. We established only the expression of ERα, not ERβ, because the level of ERβ expression is extremely low in MCF-7 cells, and the role of ERβ in breast cancer is still unclear (Vladusic *et al.*, 2000). The basal level of ERα expression is defined as ERα expression in the absence of E2 in cells cultured in phenol red-free media containing 5% charcoal-treated fetal bovine serum. Our data indicate that the basal level of ERα expression was markedly reduced (30–50%) in all of the MCF-7/Cyr61 clones, as compared with the MCF-7/V cells (Figure 3a, left and middle panels). These results indicate that Cyr61 expression is correlated with the loss of ER expression, consistent with our previous finding that Cyr61 expression is closely associated with tumor progression and ER negativity in tumor biopsies (Tsai *et al.*, 2000). It has been published recently that Cyr61 expression is associated with diagnosis of advanced diseases of breast cancer (Xie *et al.*, 2001), however, the sample number tested was relatively small. Curiously, however, the same report showed that Cyr61 expression in human breast biopsies is correlated with ER positivity (Xie *et al.*, 2001; Sampath *et al.*, 2001), even though ER expression is known to be an indicator of good prognosis for breast cancer (Brower *et al.*, 1999). Therefore, more studies are required to establish the mechanisms of Cyr61 action and its role in breast carcinomas.

We next examined whether Cyr61 promotes loss of ER function by assessing regulation of several well-documented E2-responsive genes. We tested ERα, shown to be downregulated by E2, and progesterone receptor (PgR), Cathepsin D and pS2, all of which have been shown to be upregulated by E2 in MCF-7 cells (Read *et al.*, 1989; Nardulli *et al.*, 1988; Cavailles *et al.*, 1988; Weaver *et al.*, 1988). We have previously demonstrated that the loss of PgR regulation by E2 attests for the loss of ER function (Tang *et al.*, 1996; Saceda *et al.*, 1996). Our studies were then focused on E2 regulation of ERα and PgR expression as previously described (Tang *et al.*, 1996). Although the level of ERα expression is lowered, E2 still down-regulates the expression of ERα in MCF-7/Cyr61 cells. This regulation is normally tightly controlled by E2 in the parental MCF-7 cells. Our results demonstrate that in the presence of E2, ERα expression is downregulated about 40–50% in the MCF-7/V2 cells, as compared with the untreated cells (Figure 3a, right panel). In contrast, E2 does not downregulate ERα expression in MCF-7/Cyr61 cells to the same extent as in the MCF-7/V2 cells. E2-induced downregulation of ERα in the MCF-7/Cyr61 cells was only about 10–25% as compared with the untreated cells (Figure 3a, left panel). The diminished effect of E2 on ERα expression is most probably because the basal level of the ERα expression in MCF-7/Cyr61 cells is already markedly reduced (Figure 3a, middle panel), therefore additional treatment with E2 cannot further induce a significant reduction in ERα expression (Figure 3a, right panel).

We then tested whether ER was still a functional receptor for E2 to induce upregulation of PgR gene expression in MCF-7/Cyr61 cells. Interestingly, even though ERα expression was significantly lowered in MCF-7/Cyr61 cells, E2 induced a marked upregulation in PgR mRNA expression. The increase in PgR expression was between 300% and 400%, as compared with the untreated MCF-7/Cyr61 cells (Figure 3b). As expected, E2 upregulated the levels of PgR in the MCF-7/V2 cells by about 200%. Similarly, we observed E2-induced upregulation of other E2-responsive genes, including Cathepsin D and pS2 (data not shown). These data support the notion that ERα is still a functional receptor in MCF-7/Cyr61 cells, although these cells are E2-independent and the level of ERα expression is lower than that in the parental cells. Our combined results demonstrate that Cyr61 is sufficient to confer E2-independence and antiestrogen-resistance. Interestingly, the cells transfected with Cyr61 retained E2-responsiveness and the ERα function. Moreover, we have clearly demonstrated that Cyr61 is capable of downregulating ER expression and promotes an invasive phenotype. This is consistent with our previous data which demonstrates that in breast cancer cells, Cyr61 expression correlates with the loss of ER expression, increased tumorigenicity, and the ability of cells to metastasize *in vivo* (Tsai *et al.*, 2000).

To assess the effect of Cyr61 expression *in vivo*, one vector control clone (MCF-7/V2) and four Cyr61 clones (MCF-7/C6, C9, C19, and C20) were inoculated into the mammary fat pads of 4–5 week old ovariectomized athymic nude mice. Two experimental groups (four mice per group) were assessed. One group

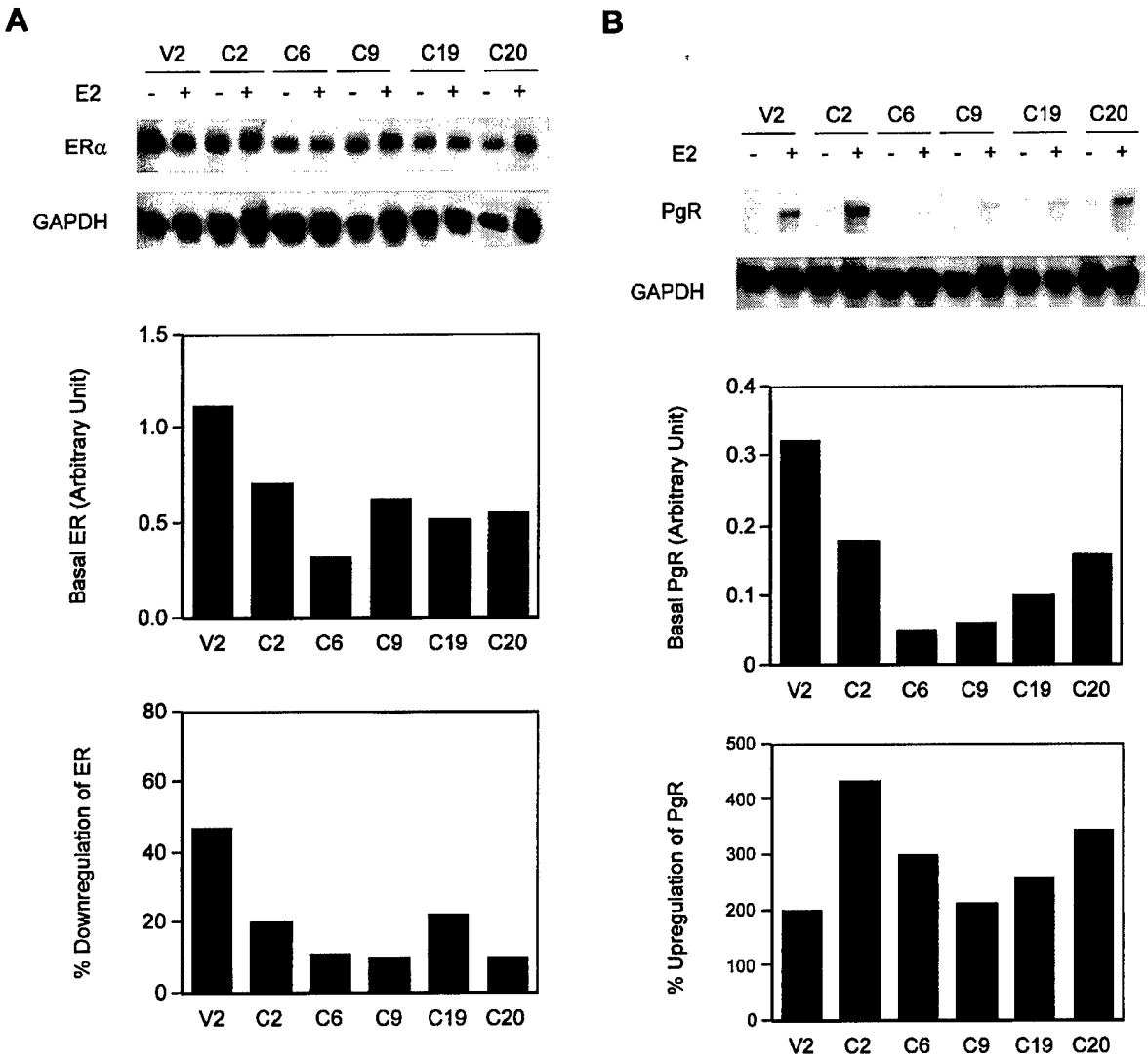

Figure 3 E2-responsive gene expression in MCF-7/Cyr61 clones. Subconfluent MCF-7/V2 and MCF-7/Cyr61 cells were cultured in media containing charcoal-stripped FBS for 4 days and treated in the presence or absence of E2 for 6 h. Total RNA was isolated, and 20 and 30 μg of total RNA were analysed respectively for the expression of (**a**) ERα and (**b**) PgR by the RNase protection assay as previously described (Tang *et al.*, 1996). Expression of ERα or PgR was normalized by GAPDH

was studied in the absence of estrogen pellets and the other in the presence of estrogen pellets. Large tumors (ranging between 0.5 cm^3 and 1 cm^3) were spontaneously developed only in those mice inoculated with MCF-7/Cyr61 clones (4/4 for clone C20 and 3/4 for other three clones) just 5 weeks after initial inoculation. Experiments were carried out for 10 weeks during which tumors were growing exponentially with time. These tumors grew independently of hormonal stimulation (Figure 4a). The MCF-7/V2 cells used as a control only developed tumors in the presence of E2 stimulation (Figure 4a). These data demonstrate indisputably that transfection of MCF-7 cells with Cyr61 promotes tumorigenesis *in vivo* in the absence of hormonal stimulation. These findings support our *in vitro* data demonstrating that the MCF-7/Cyr61 cells had a growth advantage in the absence of E2. Significantly, the data from our model system provide novel evidence which resembles many breast cancer clinical cases, showing the progression of the disease from an ER-positive and E2-dependent phenotype to an E2-independent phenotype, yet frequently without the complete loss of ER expression.

The tumors developed by the MCF-7/Cyr61 cells were excised and analysed macroscopically. The majority of the tumors appeared as firm, poorly defined masses. The lesions measured between 1 and 1.5 cm in the greatest dimension. The MCF-7/Cyr61-derived tumors demonstrated fixation to the underlying soft tissues, as well as erosion of the overlying skin. Enlargement of axillary lymph nodes was detected in the same group, whereas no macroscopic metastatic foci to visceral organs were

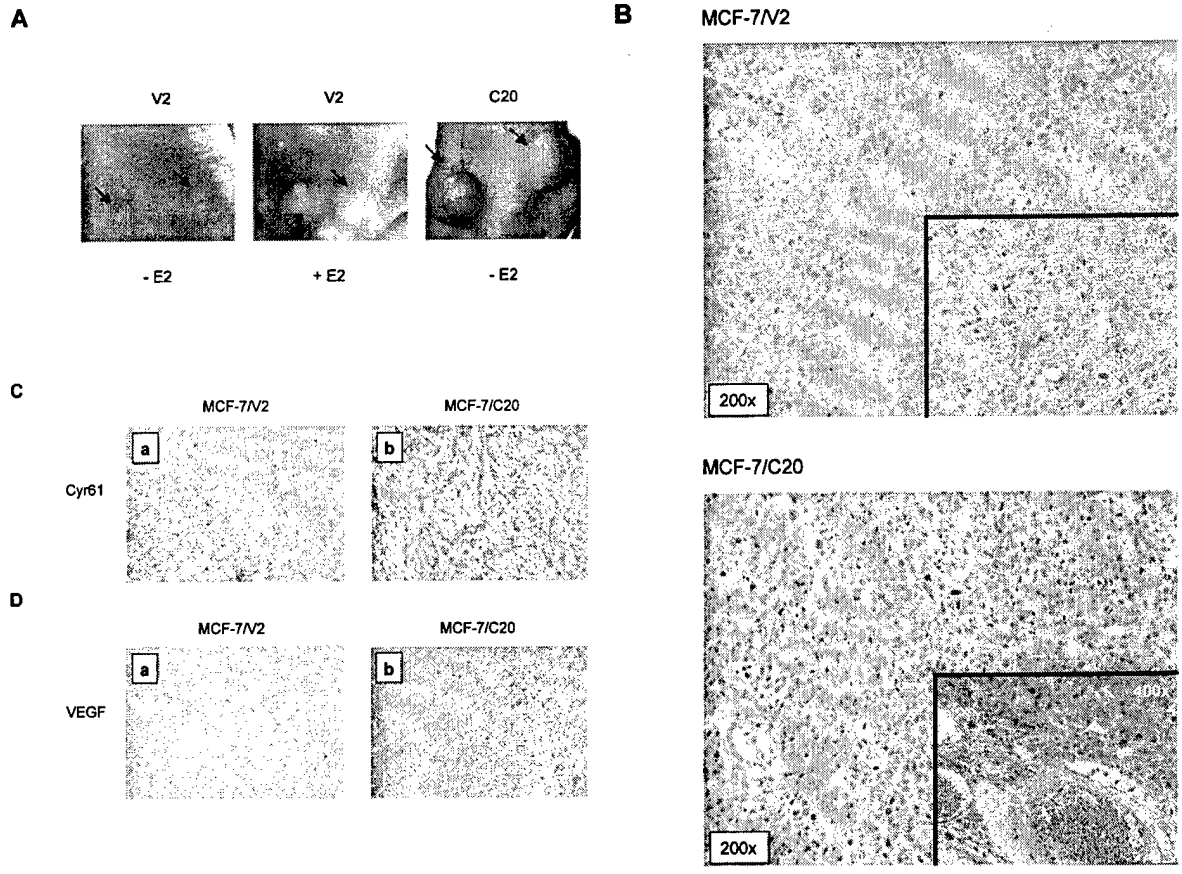

Figure 4 Expression of Cyr61 and VEGF in implanted breast tumor sections from nude mice. (**a**) Photographs of nude mice bearing human breast tumors. Tumors were developed by implanting MCF-7/V2 and MCF-7/Cyr61 cells (2×10^6 cells per site) into the mammary fat pads of 4–5 week old ovariectomized athymic nude mice with or without E2 pellets (25 mg, 90-day slow release) as previously described (Tang *et al.*, 1996). (**b**) Hematoxylin and eosin (H&E) staining of human breast tumor sections derived from MCF-7/V2 and MCF-7/Cyr61 clone (clone C20 shown). (**c**) Immunohistochemical analysis of Cyr61 expression in breast tumor sections developed from implanted MCF-7/V2 and MCF-7/Cyr61 cells (clone C20 shown) in nude mice as previously described (Tsai *et al.*, 2000). (**d**) Immunohistochemical analysis of VEGF expression in breast tumor sections derived from implanted MCF-7/V2 and MCF-7/Cyr61 cells in nude mice. Microphotographs are shown at 200× magnifications, unless otherwise specified

noticed (Figure 4a). Lymph nodes involvement in the tumors was not observed. In all of the MCF-7/Cyr61 cell groups, the tumors were characterized by histological, tightly cohesive areas of large, pleiomorphic cells with irregular nuclei and numerous mitotic figures. Multinucleated cells were observed. Neovascular formation and areas of necrotic tissues were observed (Figure 4b).

By immunohistochemical staining using an anti-Cyr61 antibody, we observed very high levels of Cyr61 expression in the tumors developed from MCF-7/Cyr61 cells. On the contrary, the levels of Cyr61 expression in tumors developed with the control MCF-7/V2 cells in the presence of E2 were very low or undetectable (Figure 4c). These data demonstrate that the expression of Cyr61 is maintained *in vivo*, and that the phenotypic changes are mediated most probably through the Cyr61 protein. The tumors developed from the Cyr61-expressing cells were evidently vascularized (Figure 4a,b). We therefore tested the expression of another angiogenic factor apart from Cyr61, the vascular endothelial growth factor (VEGF), a growth factor to stimulate neovascularization. Using immunohistochemical staining with an anti-VEGF antibody, a marked increase in VEGF expression was observed in the tumor sections derived from the MCF-7/Cyr61 cells, but not in sections derived from MCF-7/V2 tumors formed in the presence of E2 (Figure 4d). The detection of the VEGF expression by immunohistochemical staining is specific, because no background staining was observed when using the control peptide-neutralized anti-VEGF antibody (data not shown). Moreover, our results confirm a recent report showing that Cyr61 regulates VEGF expression in primary skin fibroblasts (Chen *et al.*, 2001).

Overall, the role of Cyr61 in breast cancer is still under investigation and further studies are necessary to determine the mechanism by which Cyr61 promotes breast cancer progression. We have clearly established that Cyr61 promotes tumor growth of breast cancer cells, in accordance with earlier observations, showing

that a gastric adenocarcinoma cell line RF1 becomes tumorigenic when Cyr61 is introduced in those cells (Babic *et al.*, 1998). While this work and manuscript were in progress, another study described the ability of Cyr61 to induce tumorigenicity of breast cancer and breast normal cells (Xie *et al.*, 2001). However, the study addresses neither the ER expression/function, nor the extensive biological characterization as we have presented here.

In this study, we demonstrate that expression of Cyr61 leads to E2-independence and antiestrogen resistance in MCF-7 cells for both anchorage-dependent and -independent growth. In the Matrigel matrix, Cyr61 promotes outgrowth of the MCF-7 cells in an E2-independent manner. Apparently, Cyr61 down-regulates the expression of ERα yet does not disrupt its function. Thus, Cyr61-expressing cells are still E2-responsive. We also demonstrate that overexpression of Cyr61 induces tumor formation in immunodeficient mice and promotes the expression of VEGF, an important regulator of neovascularization. Our current results further imply that Cyr61 is a downstream effector of HRG, because Cyr61 can bypass the effect of HRG and induce similar phenotypic changes of breast cancer cells as those promoted by HRG.

It has been shown that Cyr61 is an angiogenic ligand for $\alpha v\beta 3$ (Babic *et al.*, 1998, 1999). We have previously reported that a functional blocking antibody against $\alpha v\beta 3$ is capable of blocking HRG induction of the aggressive phenotypes of the breast cancer cells (Tsai *et al.*, 2000). We have proposed that Cyr61 mediates tumor growth and angiogenesis of breast cancer cells in either an autocrine or paracrine manner through its binding to the $\alpha v\beta 3$ integrin receptor. This receptor is often expressed in endothelial as well as epithelial cells. Integrin receptors have been shown to mediate cellular adhesion to the extracellular matrix, which is known to exert profound control over the cells. Although Cyr61 binds to several integrins $\alpha v\beta 3$, $\alpha IIb\beta 3$ and $\alpha 6\beta 1$, so far only the $\alpha v\beta 3$ integrin has been shown to play a major role in breast cancer tumor vascularization and progression (Meyer *et al.*, 1998). More importantly, it has been recently demonstrated that overexpression of $\alpha v\beta 3$ is a marker for poor prognosis in breast cancer (Gasparini *et al.*, 1998). We can then postulate that binding of Cyr61 to the angiogenic integrin receptor $\alpha v\beta 3$ should provide new insights into the possible mechanisms by which Cyr61 promotes breast tumorigenesis and cancer progression. We are currently investigating this avenue that will bring to light further molecular mechanisms of Cyr61 action.

Acknowledgments

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Appendix III

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SHORT REPORTS

Expression and regulation of Cyr61 in human breast cancer cell lines

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We have shown that Cyr61, an angiogenic regulator, is overexpressed in invasive and metastatic human breast cancer cells and tumor biopsies. We have further demonstrated that Cyr61 promotes acquisition of estrogen-independence and anti-estrogen resistance *in vivo* in breast cancer cells. Moreover, we have demonstrated that Cyr61 induces tumor formation and tumor vascularization *in vivo*, events mediated through the activation of the MAPK and the Akt signaling pathways. Here we investigate how Cyr61 expression is regulated in both estrogen receptor (ER)-positive and ER-negative breast cancer cells. We demonstrate that Cyr61 mRNA and protein expression is inducible by estrogen and anti-estrogens in ER-positive breast cancer cells. We show that a labile protein as well as a negative regulator might be involved in Cyr61 expression in estrogen-dependent breast cancer cells. Other important regulators of Cyr61 expression in breast cancer cells that we found are the phorbol ester TPA, vitamin D, and retinoic acid. TPA causes positive regulation of Cyr61 expression in ER-positive MCF-7 cells. Vitamin D induces a transient stimulatory effect on Cyr61 gene expression. Lastly, retinoic acid has a negative effect on Cyr61 expression and downregulates its expression in MCF-7 cells. Interestingly, most of these effects are not seen in aggressive breast cancer cells that do not express ER and express high levels of Cyr61, such as the MDA-MB-231 cells. Our results are in agreement with our knowledge that Cyr61 promotes tumor growth, and that tumor-promoting agents have a positive impact on cells that express low levels of Cyr61, such as the ER-positive breast cancer cells; however, these agents have no significant effect on cells that express high levels of Cyr61. Our findings suggest an association between increased Cyr61 expression and an aggressive phenotype of breast cancer cells.
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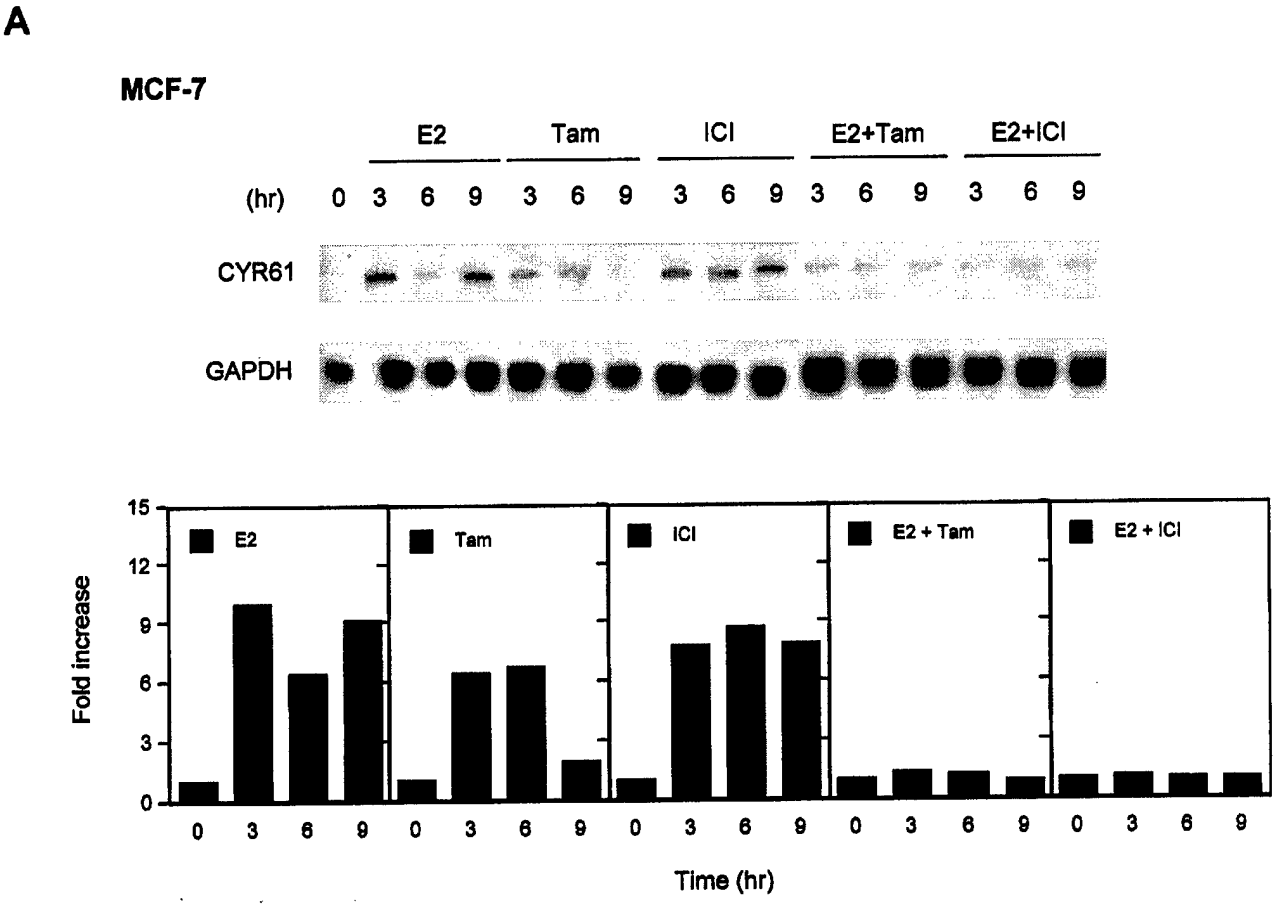
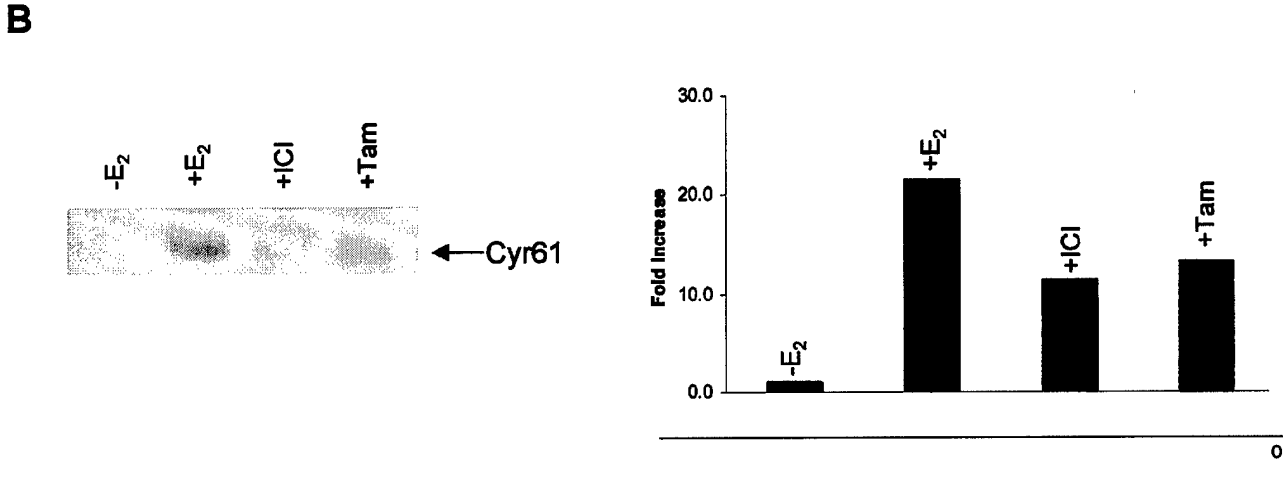
We have previously identified an angiogenic regulator, Cyr61, as a protein potentially involved in breast cancer progression (Tsai *et al.*, 2000). Cyr61 belongs to the CCN family, which consists of Cyr61, CTFG, Nov, WISP-1, WISP-2, and WISP-3 (Lau and Lam, 1999). These structurally conserved proteins share four modular domains with similar sequence homologies (Lau and Lam, 1999). Cyr61 is a cysteine-rich, heparin-binding protein that is secreted and associated with the cell surface and the extracellular matrix (ECM) (Yang and Lau, 1991; Kirreva *et al.*, 1997). Cyr61 mediates cell adhesion, induces cellular migration, enhances growth factor-induced DNA synthesis in fibroblasts and endothelial cells, stimulates chemotaxis of fibroblasts and endothelial cells, and increases chondrogenesis in mesenchymal cells (O'Brien and Lau, 1992; Kireeva *et al.*, 1996, 1997, 1998; Frazier *et al.*, 1996; Wong *et al.*, 1997; Kolesnikova and Lau, 1998; Babic *et al.*, 1998), possibly through its binding to integrin receptors, such as $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha IIb\beta 3$, and $\alpha 6\beta 1$ (Kireeva *et al.*, 1998; Jedsadayanmata *et al.*, 1999; Chen *et al.*, 2000; Grzeszkiewicz *et al.*, 2001). Most significantly, expression of Cyr61 enhances neovascularization and tumor formation of human tumor cells in immunodeficient mice (Babic *et al.*, 1998, 1999; Xie *et al.*, 2001).

We have shown that Cyr61 is overexpressed in invasive and metastatic human breast cancer cells and tumor biopsies (Tsai *et al.*, 2000). In addition, we demonstrated that Cyr61 function is necessary for heregulin (HRG)-mediated chemomigration of breast cancer cells (Tsai *et al.*, 2000). We further determined that Cyr61 is sufficient to promote breast cancer cells to bypass their normal estrogen requirement for growth, to induce progression of breast cancer cells to more aggressive and invasive phentoypes *in vitro*, and to induce tumor formation *in vivo* (Tsai *et al.*, 2001, manuscript submitted). To understand better the mechanism of Cyr61 action in promoting breast cancer progression, it is important to investigate how Cyr61 expression is regulated in breast cancer cells.

It is well known that Cyr61, an immediate-early gene, is transcriptionally activated by serum, platelet-derived growth factor (PDGF), transforming growth factor-$\beta 1$ (TGF-$\beta 1$), and basic fibroblast growth factor (bFGF) in fibroblasts and neuronal cells (Lau and Nathans, 1987; O'Brien *et al.*, 1992; Ryseck *et al.*, 1991; Bunner *et al.*, 1991). Other cytokines, such as tumor necrosis factor α (TNFα) and interleukin-1 (IL-

1) also strongly elevate the expression of Cyr61 (Scheutze *et al.*, 1998). Cyr61 is differentially expressed by muscarinic acetylcholine receptors (mAChRs) in the brain (Albrecht *et al.*, 2000), and by factor VIIa and thrombin in human fibroblasts (Pendurthi *et al.*, 2000). Phorbol ester 12-*O*-tetradecanoyphorbol-13-acetate (TPA) mediates Cyr61 expression in the liver (Nathans *et al.*, 1988). Incidentally, others and we have found expression of Cyr61 involving protein kinase C (PKC) and the MEK/MAPK pathways (Chung and Ahn, 1998; Inuzuka *et al.*, 1999; Albrecht *et al.*, 2000; Tsai *et al.*, 2001, manuscript submitted). Cyr61 is also vitamin D-responsive in human osteoblasts and osteosarcoma cell lines (Scheutze *et al.*, 1998). Induction of Cyr61 may be attenuated by glucocorticoids in murine fibroblasts (Smith and Herschman, 1995). Moreover, previous studies showed that murine Cyr61 is inducible by estrogen (E2) and tamoxifen (Tam) in the uterus of ovariectomized rats (Rivera-Gonzalez *et al.*, 1998). However, it is not clear how Cyr61 is regulated in human breast cancer, except it has been indicated that Cyr61 is E2-inducible, and that Tam inhibited its





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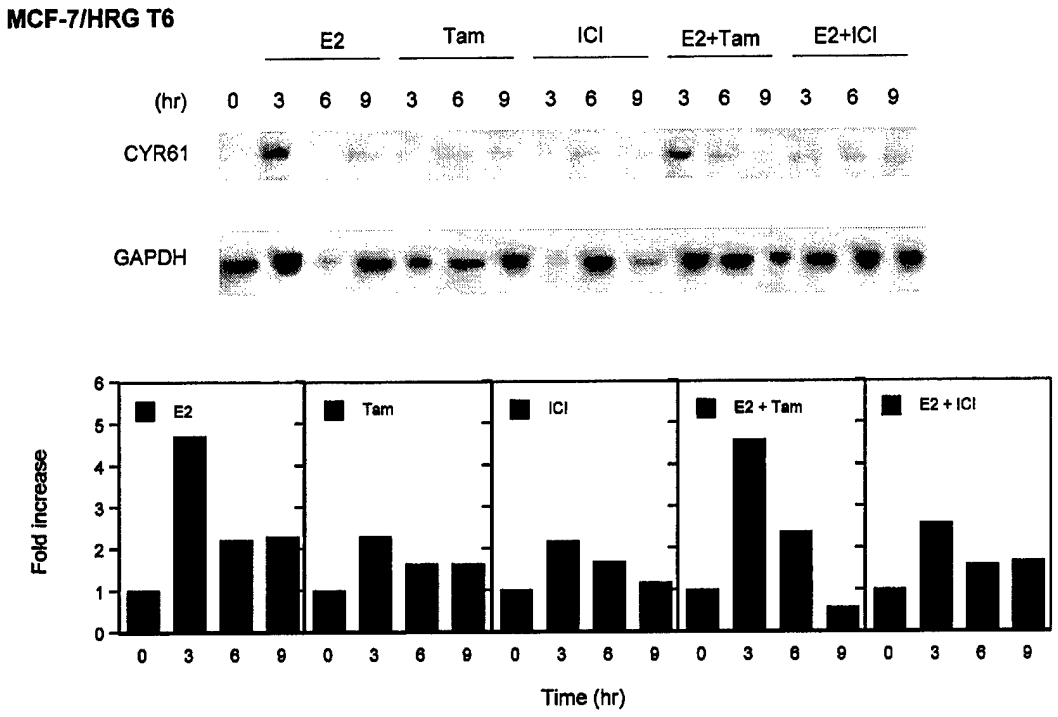
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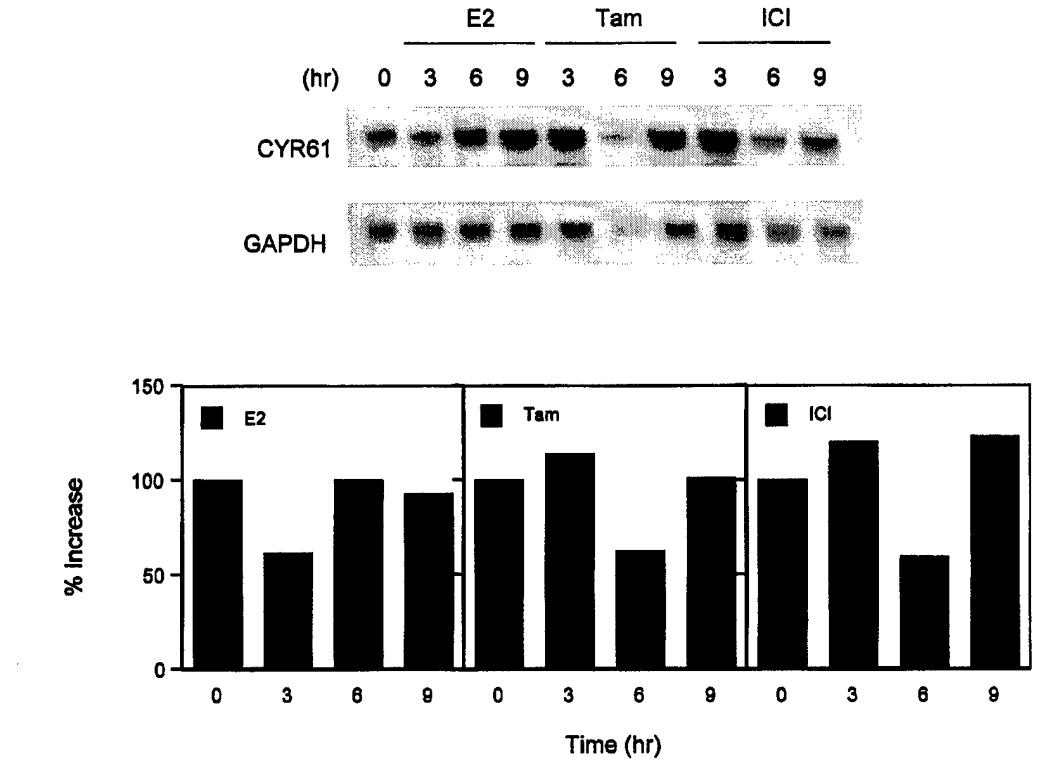
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expression in human breast cancer cells (Xie *et al.*, 2001).

We have recently shown that Cyr61 is expressed in breast cancer cells, and that enhanced expression of Cyr61 correlates with lack of ER expression and with advanced breast cancer diseases (Tsai *et al.*, 2000). Here we investigate in more detail how Cyr61 expression is regulated in both ER-positive and ER-negative breast cancer cells. To determine whether Cyr61 expression was regulated by estrogen, anti-estrogens (anti-E2), or both, in breast cancer cells that express or do not express ER, the levels of Cyr61 mRNA were measured in a time-dependent manner by RNase protection assays. Cells were grown in phenol red-free media containing FBS depleted of estrogenic compounds by charcoal adsorption, and treated in the presence or absence of E2.

As shown in Figure 1a, the mRNA levels of Cyr61 in MCF-7 cells under the condition of estrogen depletion was very low or nearly undetectable. In contrast, E2 markedly increased the expression of Cyr61 mRNA. Upregulation of Cyr61 was observed as early as 3 h after E2 treatment, as a 6- to 10-fold increase (Figure 1a, top panel). The Cyr61 transcript, although slightly reduced after 9 h of treatment, was sustained for up to 72 h without significant decreases (data not shown).

To provide a better understanding of the possible mechanism of anti-estrogenic effects, two distinct anti-estrogens, Tam and ICI 182, 780 (ICI), were used. Tam functions as an antagonist as well as a partial agonist by binding to the ligand-binding domain, thus blocking transactivation of the AF-2 domain but not the AF-1 of the ER. ICI, a pure anti-estrogen, displays solely antagonistic effects by binding to the ER, disrupting

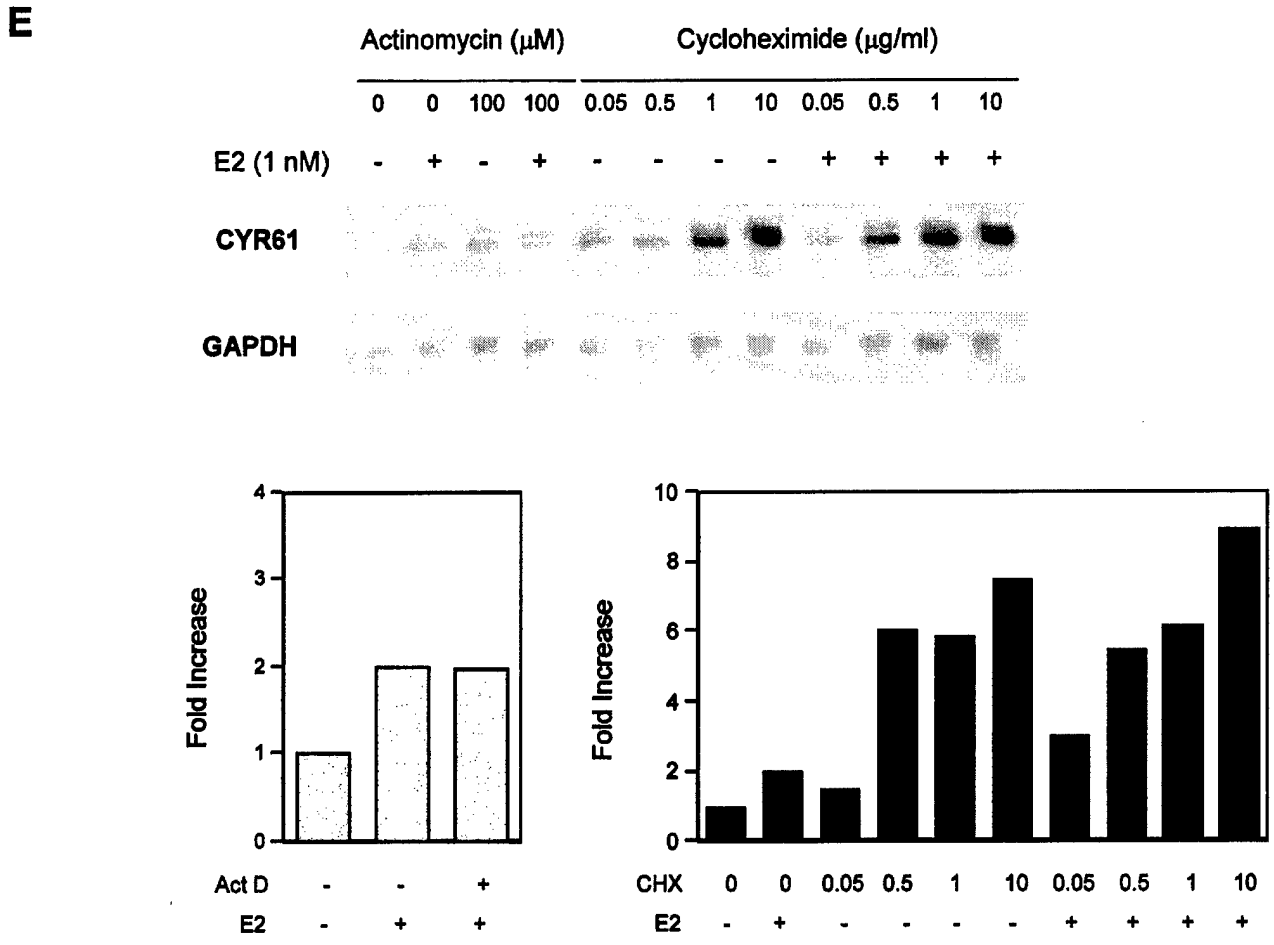
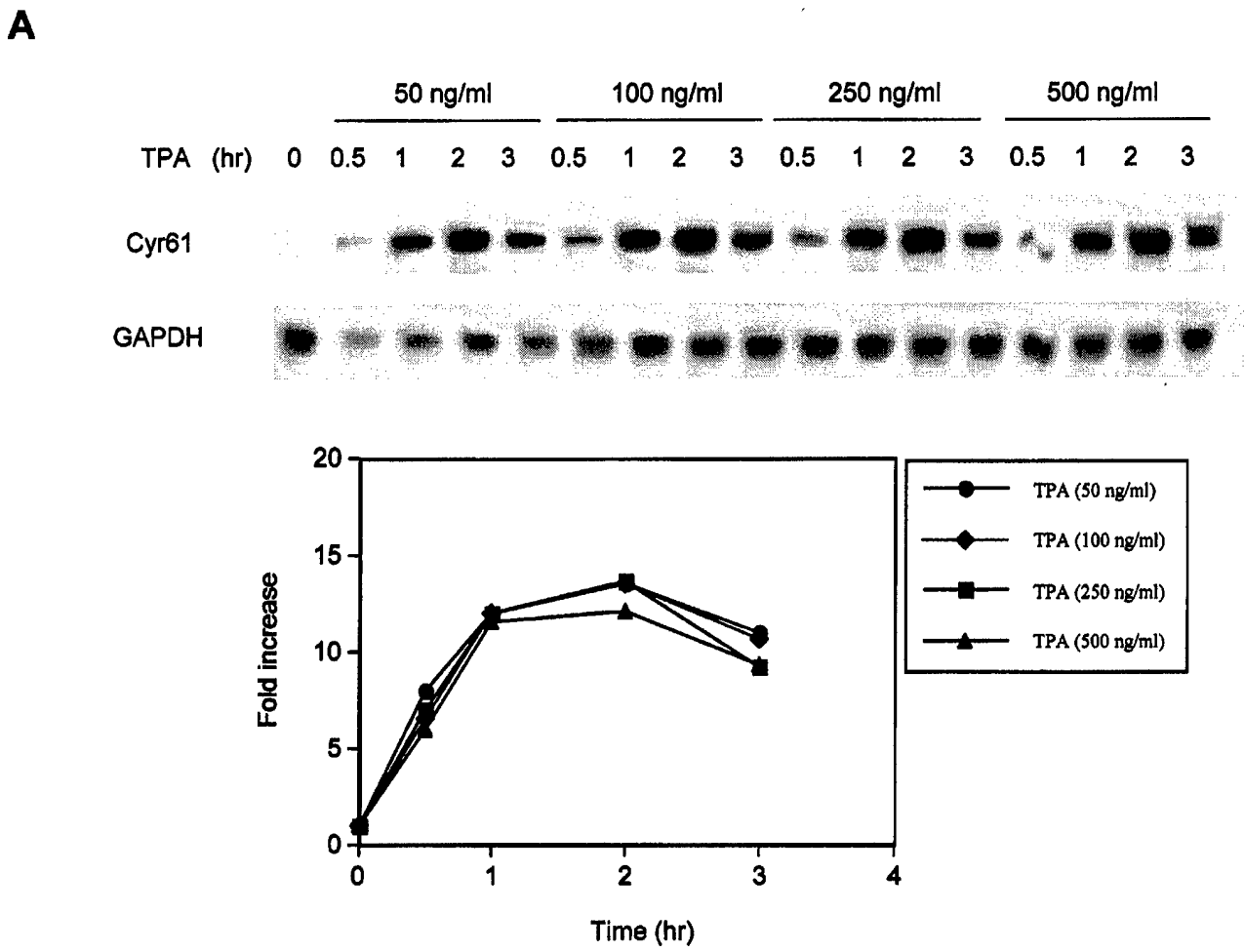


Figure 1 Effect of estrogen, anti-estrogen, actinomycin D, and cycloheximide on Cyr61 expression in breast cancer cells. Breast cancer cells, (**a**) MCF-y, (**b**) MCF-7, (**c**) MCF-7/HRG clone T6, and (**d**) MDA-MB-231, were cultured in E2-depleted media for 4 days. For (**a**,**c**,**d**), total RNA was isolated at 0, 3, 6 and 9 h after treatments with E2 (1 nM), Tam (100 nM), ICI (100 nM), the combination of E2 and Tam, and the recombinant of E2 and ICI. The RNA was analysed for Cyr61 expression by the RNase protection assay as described previously (Tsai *et al.*, 2000). For (**b**), conditioned media was collected after 24 h of E2, Tam or ICI treatment and concentrated 50×. Western blot analysis was then performed using 20 μl of the concentrated media. (**e**) MCF-7 cells were cultured in E2-depleted media and incubated with a control vehicle, and with E2 (1 nM) in the presence or absence of actinomycin D (Act-D; 100 nM) or cycloheximide (CHX; 0.05, 0.5, 1, and 10 μg/ml) for 6 h. Total RNA was isolated and analysed by the RNase protection assays as above. GAPDH is used as a control of RNA loading. Data were quantified by densitometry and normalized to the level of GAPDH. Similar results were obtained from at least four independent experiments



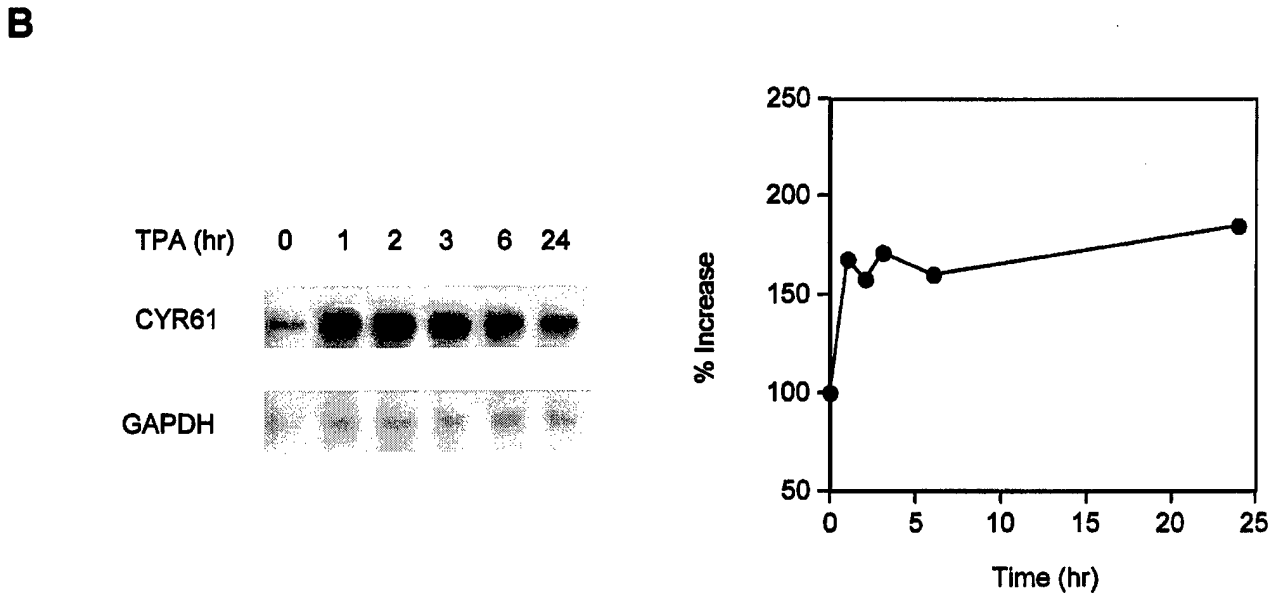


Figure 2 Effect of TPA on Cyr61 mRNA expression in breast cancer cells. (**a**) MCF-7 cells were treated with TPA (50, 100, 250, and 500 ng/ml) for 0, 0.5, 1, 2, and 3 h. Total RNA was isolated and analysed by the RNase protection assay. (**b**) MDA-MB-231 cells were treated with 50 ng/ml TPA for 0, 1, 2, 3, 6, and 24 h. Total RNA was isolated and analysed as described above. Data were quantified by densitometry and normalized to the level of GAPDH. Similar results were obtained from at least three independent experiments

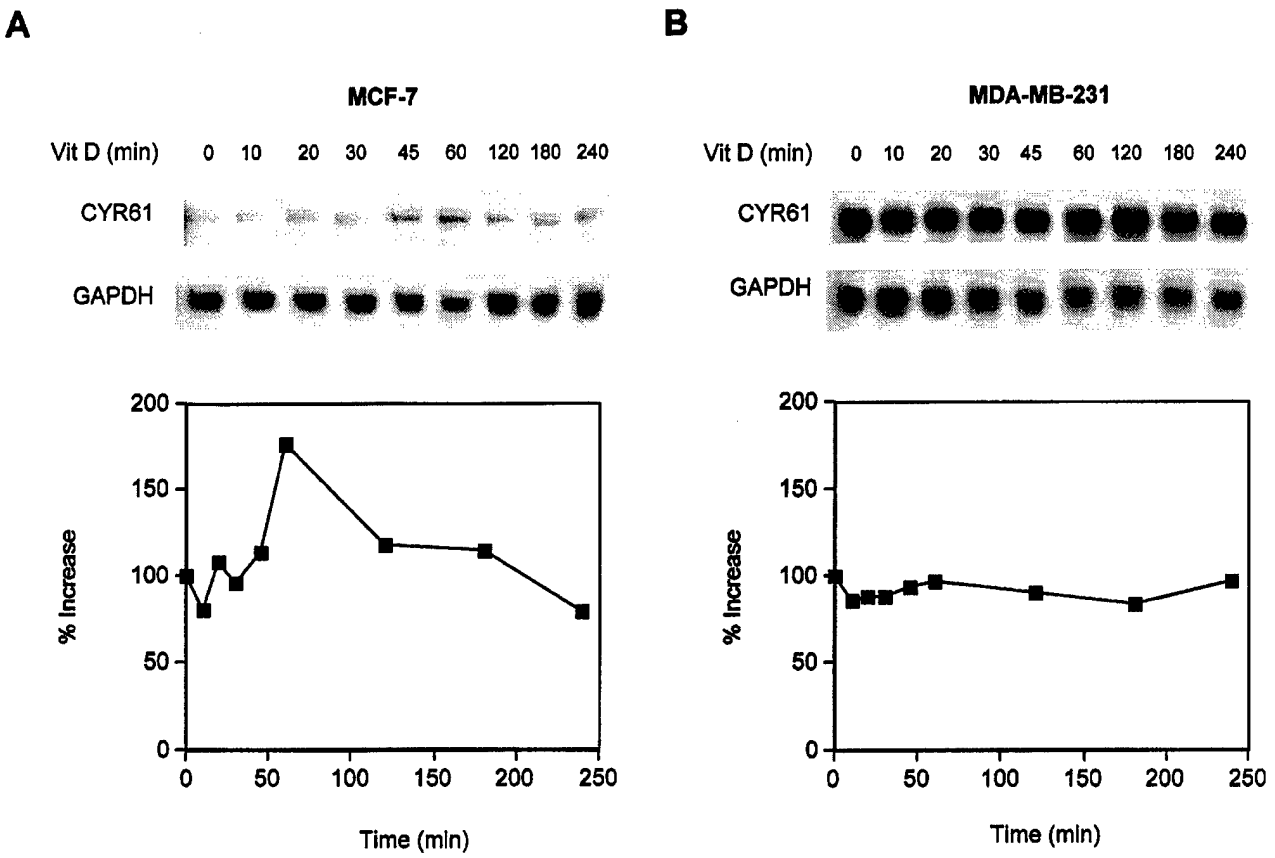


Figure 3 Effect of vitamin D on Cyr61 mRNA expression in breast cancer cells. (a) MCF-7 cells and (b) MDA-MB-231 cells were treated with vitamin D (10 mM) for 0, 10, 20, 30, 45, 60, 120, 180, and 240 min. Total RNA was isolated and analysed for Cyr61 mRNA levels by the RNase protection assays. Data were quantified by densitometry and normalized to the level of GAPDH. Similar results were obtained from at least three independent experiments

the nuclear translocation of the ER, and inducing rapid degradation of the ER (MacGregor and Jordan, 1988). We examined the effects of the anti-estrogens on E2 induction of Cyr61 expression. Cells were treated with E2 alone, or with the combination of E2 and anti-E2. Both anti-estrogens exerted antagonistic effects on E2.

Interestingly, treatment with either anti-estrogen on MCF-7 cells caused a marked upregulation of Cyr61 mRNA (Figure 1a, top panel). Tam induced the expression of Cyr61 mRNA by a sixfold increase after 3 h of treatment, and then its expression was reduced to the basal level after 9 h. On the other hand, ICI, a pure E2 antagonist, induced rapid Cyr61 upregulation kinetics (Figure 1a, top panel), similar to that observed for E2; that is, the accumulated Cyr61 mRNA was seen as early as 3 h after treatment and maintained for up to 72 h (data not shown). Interestingly, the combination of E2 with Tam or ICI abrogated the expression of Cyr61. This effect can be due to a change in receptor occupancy and the modification of the ER pockets that each of these compounds interfere with. Quantification of Cyr61 protein expression was performed using densitometry (Figure 1a, bottom panel).

Moreover, we demonstrated that Cyr61 protein expression was also upregulated (over 10–20-fold) by E2, Tam and ICI in MCF-7 cells (Figure 1b). Quantification of the Cyr61 expression was performed using densitometry. These cells were grown in phenol red-free media containing FBS depleted of estrogenic compounds by charcoal adsorption for 4 days and treated with 10^{-9} M E2 for 24 h. The media was then collected and concentrated 50 times. Western blot analysis was performed as previously described (Tsai *et al*., 2000). While writing this manuscript, these data were confirmed recently by Sampath *et al*., 2001.

We then examined the expression of Cyr61 in E2-independent and anti-E2-resistant breast cancer cells, such as HRG-transfected MCF-7 clones (MCF-7/HRG) (Tang *et al*., 1996) and MDA-MB-231. We first determined whether E2-induced Cyr61 expression is mediated in one of the MCF-7/HRG clones (clone T6), all of which express high levels of HRG and moderate levels of Cyr61. Both E2 and anti-E2 transiently induced Cyr61 expression in MCF-7/HRG cells. The highest expression was observed after 3 h of E2 treatment, returning to the basal level shortly after 9 h of treatment. Although the MCF-7/HRG cells acquired an E2-independent phenotype both *in vitro* and *in vivo* (Tang *et al*., 1996), it appears that these cells respond slightly to E2 in regulating Cyr61

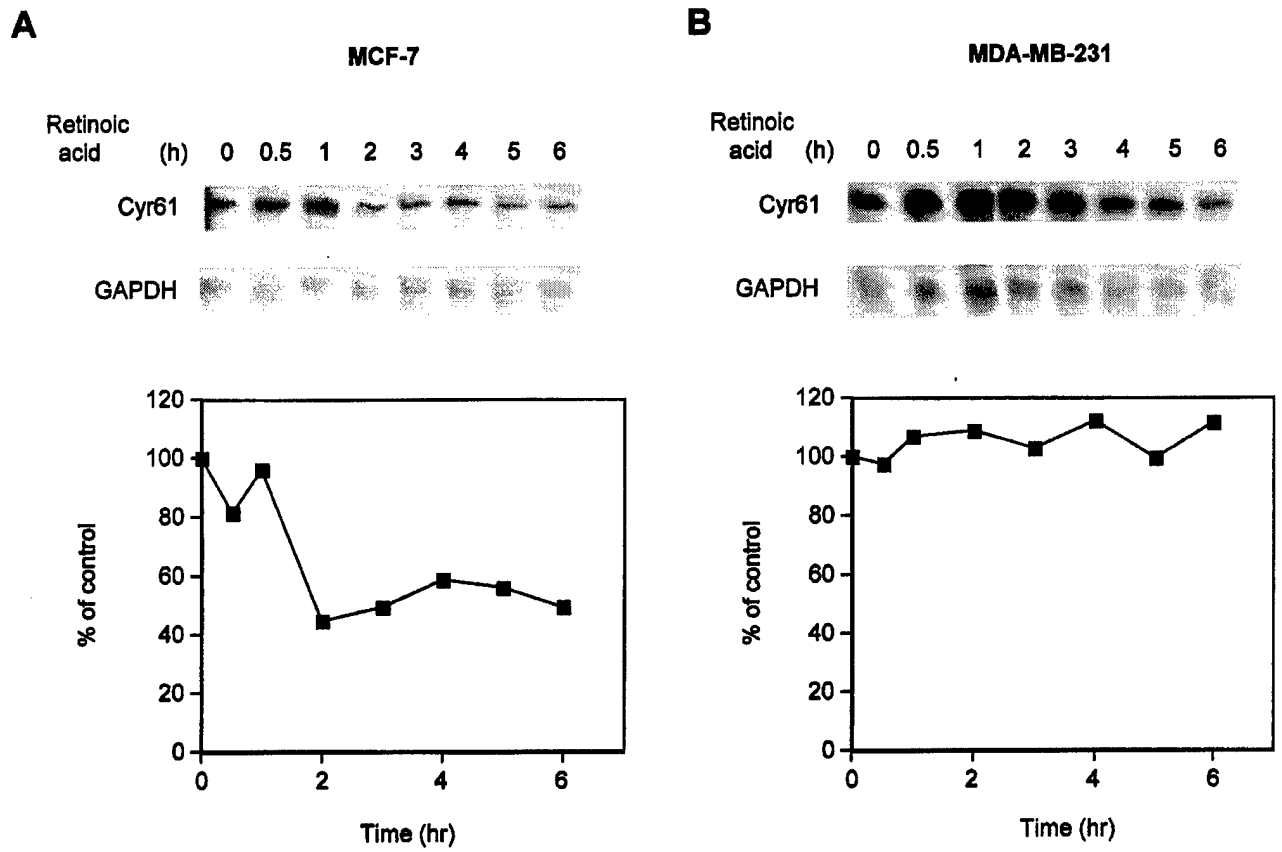


Figure 4 Effect of retinoic acid on Cyr61 mRNA expression in breast cancer cells. (**a**) MCR-7 cells and (**b**) MDA-MB-231 cells were treated with 10 nM all trans-retinoic acid for 0, 0.5, 1, 2, 3, 4, 5, and 6 h. Total RNA was isolated and analysed for Cyr61 mRNA levels by the RNase protection assay. Data were quantified by densitometry and normalized to the level of GAPDH. Similar results were obtained from at least three independent experiments

expression, however with different kinetics as compared to wild-type MCF-7 cells (Figure 1c). Furthermore, the level of Cyr61 induction by E2 and anti-E2 in the MCF-7/HRg cells was 2–3-fold lower as compared to wild-type MCF-7 cells. Moreover, while ICI blocked E2-mediated Cyr61 expression by 50 to 70% as early as 3 h after treatment, Tam showed an apparent delayed blockage of 50% after 6 h of treatment and a complete blockage after 9 h of treatment (Figure 1c). Thus, the effects of Tam and ICI on E2 induction of Cyr61 expression appear to result from distinct pathways in MCF-7/HRG cells, possibly because these cells become resistant to both Tam and ICI, while only Tam has agonistic effect and induces tumor growth *in vivo* (Atlas *et al.*, 2001, manuscript submitted). Quantification of the Cyr61 expression is shown in the bottom panel of Figure 1c using densitometry.

Next we examined the effect of E2 on Cyr61 expression in breast cancer cells that do not express ER. As expected and shown in Figure 1d, E2 and anti-E2 no longer mediate the expression of Cyr61 in MDA-MB-231 cells, which express high levels of endogenous Cyr61. This finding is consistent with the phenotypes of MDA-MB-231, which is ER-negative, E2-independent, anti-E2-resistant, highly invasive, and metastatic *in vivo*. Quantification of the Cyr61 expression was performed using densitometry and is shown in Figure 1d, bottom panel.

Together, our results indicate that the fold induction of Cyr61 by E2 and or anti-E2 coincides with the endogenous levels of Cyr61 expression and is inversely correlated with the levels of ER expression in breast cancer cells as shown in Figure 1a,c,d. Here we demonstrate that E2, a tumor-promoting hormone, highly induces the expression of Cyr61 in breast cancer cells that express ER. In cells that acquire E2-independence but still express ER, such as the MCF-7/HRG cells, E2 induces Cyr61 to a much lower extent. In contrast, in cells that are very aggressive, do not express ER, and express high levels of Cyr61, E2 fails to further induce Cyr61 expression. These results are consistent with the hypothesis that Cyr61 expression correlates with the aggressiveness of the breast tumors, which in turn correlates with the lack of ER expression (Tsai *et al.*, 2000).

Our data clearly demonstrate that Cyr61 is an E2-inducible gene in breast cancer cells. To investigate further whether transcription is involved in the E2-mediated increase in Cyr61 mRNA steady-state levels, MCF-7 cells were depleted of E2-like compounds and

incubated with E2 (1 nM) and actinomycin-D (100 nM), which is an RNA synthesis inhibitor. As shown in Figure 1e, we demonstrate that E2 stimulates Cyr61 expression in MCF-7 cells (Figure 1e), and that actinomycin-D treatment (100 μM) has no effect on E2-mediated Cyr61 expression, indicating that *de novo* RNA synthesis is not required in E2 induction of Cyr61. The fact that actinomycin-D upregulates the mRNA levels suggests that although the *de novo* RNA synthesis is inhibited there is upregulation of Cyr61. If so, *de novo* of Cyr61-mRNA cannot account for that. It is therefore possible that the upregulation is due to the increased stability of the RNA, and the inability of protein(s) that may be involved in the recycling of the mRNA in question are not transcribed. This is supported by the fact that when protein synthesis is inhibited there is also upregulation of mRNA, supporting the same possible mechanism, or in the later case the relief of repression. It is also possible that E2 increases the stability of the mRNA. We then determined whether *de novo* protein synthesis is necessary for the induction of Cyr61 by E2. MCF-7 cells were depleted of E2-like compounds prior to the treatment with E2 in the presence or absence of a protein synthesis inhibitor, cycloheximide. As shown in Figure 1e, the stimulatory effect of E2 was not blocked by cycloheximide. Interestingly, cycloheximide treatments markedly increased the basal level of Cyr61 expression and further increased E2-induced Cyr61 mRNA accumulation by 6–7-fold in a dose-dependent manner. These results indicate that a rapid accumulation of Cyr61 mRNA involved a labile protein(s), and that cycloheximide prevented the *de novo* protein synthesis of a negative regulator of Cyr61. Our results are consistent with the data by Sampath *et al.*, 2001 that cycloheximide does not block E2-regulated Cyr61 expression. However, we showed that cycloheximide alone regulated Cyr61 expression. In addition, our results clearly demonstrate the increase in Cyr61 expression in the presence of cycloheximide at three different concentrations in the presence or absence of physiological concentration of E2 (1 nM), whereas their data have only one concentration of cycloheximide and a 10 times higher concentration of E2 (10 nM). The discrepancy between our findings and Sampath *et al.* (2001) could be due to the known clonogenicity of MCF-7 cells. In addition, studies with actinomycin-D or cycloheximide alone were not provided in the above-cited publication. We have followed up the E2 effect on Cyr61 expression, and the Cyr61 mRNA level was sustained up to 72 h without significant decrease.

It is known that nuclear steroid/thyroid/retinoid receptors and c-*erbB* membrane receptor tyrosine kinases control epithelial cell growth and differentiation. Retinoic acid receptor (RAR) can dimerize with the vitamin D receptor (VDR), the glucocorticoid receptor, or the thyroid receptor (Schneider *et al.*, 1999). Molecular interactions between RARs and c-*erbB2*, ER and c-*erbB2*, and between ER and RARα have been reported (Flicker *et al.*, 1997; Schneider *et al.*, 1999). Moreover, these nuclear receptors and protein kinase signaling, such as protein kinase A (PKA) and protein kinase C (PKC), communicate with each other, perhaps via cross-talk regulatory factors (Schneider *et al.*, 1999). It is well documented that retinoid signaling via retinoic acid (RA) and retinoid X receptors (RARs and RXRs) regulates mammary epithelial cell growth, differentiation, morphogenesis, and apoptosis (Glass *et al.*, 1997). Therefore, we next investigated whether TPA, vitamin D, and retinoic acid (RA) play any role in the regulation of Cyr61 expression in breast cancer cells.

TPA is an effective regulator of growth of many different cell types. It activates PKC, which plays a key role in the control of many signaling pathways involved in growth, differentiation, and cellular transformation. Previous studies have shown that Cyr61 is induced by phorbol ester TPA in fibroblasts (Nathans *et al.*, 1988). It has been suggested that Cyr61 may involve PKC pathways in preadipocyte cells (Inuzuka *et al.*, 1999). Since activation of the PKC pathways has been shown to be a possible mechanism in the acquisition of drug resistance and of aggressive behavior in breast cancer cells (Bowden *et al.*, 1999), we tested the effects of TPA on the expression of Cyr61 in breast cancer cells. As shown in Figure 2a, TPA induces upregulation of Cyr61 expression (mRNA) in MCF-7 cells in a time-dependent manner, in any dose used (from 50 ng/ml to 500 ng/ml). Cyr61 transcripts were seen to increase rapidly after 30 min of treatment with TPA, reached the peak level after 2 h of treatment (a 15-fold increase), and were maintained at that high level longer than 24 h (data not shown). Interestingly, TPA induced the expression of Cyr61 expression in MDA-MB-231 cells by about 1.5-fold and this is sustained up to 24 h (Figure 2b). These results indicate that activation of the PKC pathways may be involved in the upregulation of Cyr61, resulting in the progression of breast cancer cells.

The active metabolite of vitamin D, 1,25-dihydroxyvitamin D3 (1,25-$(OH)_{2D3}$ and its several analogues are novel putative anticancer agents, with their ability to induce growth inhibition, differentiation, and apoptosis in tumor cells. 1,25-$OH)_{2D3}$ binds to the nuclear VDR with high affinity and elicits its action regulating gene expression in target cells by binding to vitamin D-responsive elements (Haussler *et al.*, 1995). Cyr61 mRNA steady-state levels have been shown to be stimulated by 1,25-$(OH)_{2D3}$ in osteoblasts (Scheutze *et al.*, 1998). Moreover, a close correlation between VDR abundance and cell proliferation rate has been shown in MCF-7 breast cancer cells, HL-60 myeloblastic cells, and several leukemia cell lines (Folgueira *et al.*, 2000). Thus, we investigated whether Cyr61 expression in breast cancer cells could also be mediated by 1,25-$(OH)_{2D3}$. As shown in Figure 3a, 1,25-$(OH)_{2D3}$ exerts rapid and transient induction of Cyr61 steady-state mRNA levels in MCF-7 cells. In these cells, Cyr61 expression was upregulated by 1,25-$(OH)_{2D3}$ after 1 h of treatment, an effect that rapidly declined to basal levels after 2 h of treatment. By contrast, 1,25-$(OH)_{2D3}$ has no effect on Cyr61 expression in MDA-

MD-231 cells (Figure 3b). It is possible that the level of the VDR is different between ER-positive and ER-negative breast cancer cells (Agadir *et al.*, 1999; Escaleira and Brentani, 1999). More likely, the extent of transcriptional co-activators and/or co-repressors, and the ligand availability of heterologous steroid hormone receptors, such as RAR and RXR, may determine the diverse function of VDR in breast cancer cells. In addition, 1,25-$(OH)_{2D3}$ has been shown to downregulate ER in MCF-7 cells (Nolan *et al.*, 1998), which may cause the transient, low-level of induction of Cyr61 by 1,25-$(OH)_{2D3}$. Our results indicate that inhibitory pathways of vitamin D may involve regulation and participation of certain growth factors, such as Cyr61. The inhibitory pathways of Vitamin D may involve regulation and participation of growth factors, such as Cyr61. Since Vitamin D transiently regulates the expression of Cyr61 in MCF-7 cells (Figure 3a) and does not regulate its expression in MDA-MB-231 cells (Figure 3b), it is conceivable that Cry61 plays little or no role, in the development of Vitamin D resistance. Since all trans-retinoic acid is a potent regulator of growth of cancer cells, and RA exerts a growth inhibitory effect on ER-positive but not on ER-negative breast cancer cells (Sheikh *et al.*, 1994; Srivastava *et al.*, 1999), and since Cyr61-over-expressing breast cancer cells acquire a growth advantage, we studied whether Cyr61 was an RA-targeted gene in MCF-7 cells. MCF-7 cells were treated with tRA in a time- and dose-dependent manner. Interestingly, Cyr61 expression was rapidly down-regulated by RA in MCF-7 cells. This inhibitory effect was evident at 2 h, at which time Cyr61 expression was decreased to 40–45%, as compared to the control (Figure 4a). On the other hand, RA has no effect on the Cyr61 level in MDA-MB-231 cells (Figure 4b). These results are consistent with the notion that RA exerts growth-inhibitory effects on breast cancer cells by ER-mediated enhancement of RAR levels, and possibly via an ER-dependent downregulation of growth-promoting factor(s).

In summary, we have demonstrated that Cyr61 is regulated by E2, and that anti-estrogens can block this induction in MCF-7 cells. Interestingly, we determined that a possible negative regulator of Cyr61 is present in MCF-7 cells. By contrast, the effects are different when Cyr61-overexpressing cells are treated with these agents. In addition, we demonstrated that both TPA and vitamin D upregulate the expression of Cyr61 in breast cancer cells that express high levels of ER, such as MCF-7 cells. In contrast, the differentiating agent RA inhibits the expression of Cyr61 in MCF-7 cells. All of these results are consistent with our previous findings that Cyr61 promotes breast cancer cells and tumor growth, and that tumor-promoting agents have a positive impact on cells in which Cyr61 expression is low, whereas these agents have no significant effect on cells that express high levels of Cyr61. On the other hand, downregulation of the growth-promoting activity of Cyr61 by RA may account for the ability of RA to induce growth inhibition and apoptosis in breast cancer cells.

In conclusion, we demonstrate that regulation of Cyr61 is involved in breast cancer progression, and that Cyr61 should be considered as a new target for therapy. We have demonstrated that Cyr61 is highly expressed in breast carcinomas and that it induces the progression of breast cancer *in vitro* and *in vivo*. We are therefore currently investigating the mechanisms that account for Cyr61 induction of breast cancer progression.

Acknowledgments

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Appendix IV

centrations was used as a positive control. After 48 hours, cellular proliferation was assessed by measuring the conversion of formazan dye from a tetrazolium salt by metabolically active cells. Both cell lines responded to both lovastatin and RYR extract with significant inhibition of proliferation ($p < 0.01$). We conclude therefore Chinese Red Yeast Rice has the potential to have anticancer activity when used as a dietary supplement.
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131 Pharmacological actions of a Chinese herbal formula used for seasonal allergic rhinitis (SAR)
GB Lenon, CCL Xue, CG Li
Presenting Author: GB Lenon
A Chinese herbal medicine formula has been proven its effectiveness in relieving symptoms of seasonal allergic rhinitis SAR during randomised clinical trial (Xue et al., 2000). However, the mechanism of the actions of this SAR formula (SARF) has not yet been elucidated. In this study, we investigated the effect of SARF on responses induced by various agents *in vitro*. In isolate tracheal preparations from rat or guinea-pigs, the responses to acetylcholine (10 μM), carbachol (1μM), substance P (0.1-10μM), 5-HT (1μM), prostaglandin E2, leukotriene C4 or histamine (0.1-30μM) were not significantly affected by SARF (0.04-1.0mg/ml). In contrast, contractions elicited by compound 48/80 (25μg/ml) in both tissues were significantly inhibited by SARF. The responses in the presence of SARF (0.4 mg/ml) were 62.6±8.6% (n=14, rat) and 36.3±9.2% (n=8, guinea-pig) compared to the control responses (91±8.6%, n=14, rat and 59.6±7.6%, n=8, guinea pig, respectively). In isolated rat aortic ring preparations, responses to endothelium dependant and independent relaxants acetylcholine and nitric oxide (NO) donor, sodium nitroprusside (SNP) respectively were not significantly affected by SARF. However, relaxations to L-arginine in lipolysaccharide-treated and endothelium denuded preparations were significantly inhibited by SARF. The maximal responses to L-arginine in the presence of SARF (0.4 mg/ml) were reduced to 17.9±4.1% (n=5) compared with the control response 92.5±5.7% (n=12). In addition, the release of leukotriene B4 (LTB4) induced by calcium ionophore in porcine neutrophils was also significantly inhibited by SARF (the release of LTB4 was 82.7±25.9 ng (n=4) in the presence of SARF (1ug/ml-1001ug/ml) compared with the control 142.8±14.2 ng (n=8). These findings indicate that SARF may have multiple pharmacological actions including the inhibitions of inducible NO synthase and the release of inflammatory mediators from target cells. Xue CCL, Thien FCK, Jamison J & Zhang JJS. (2000). Allergy and Clinical Immunology International, Suppl 2, 73.
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132 The effects of cold and heat property herbal formula on collagen-induced arthritis in rats
Shao Li, Yongyan Wang, Yinqi Hu, Aiping Lu
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It has been known that rheumatoid arthritis can be treated by two opposite approaches with heat or cold property herbal formula in Chinese medicine. This study is aimed to explore the differences and mechanisms of both approaches in collagen induced arthritis (CIA) rats. Qingluo Yin (QLY) is aimed to expel the pathogenetic heat with cold property in herbal formula, and consists of Radix sophorae flaverscentis, Cortex phellodendri, Caulis sinomenii, and Rhizoma dioscoreae hypoglaucae. Wenluo Yin (WLY) is aimed to expel the pathogenetic cold with heat property in herbal formula, and consists of Radix aconiti lateralis preparata, Rhizoma atractylodis macrocephalae, Ramulus cinnamomi, and Herba selaginellae. The 60 Wistar rats were from China Academy of Medical Sciences, and divided into three groups, control, QLY, and WLY, with 20 in each group. The CIA was induced by immunization of emulsified collagen II and complete adjuvant. The TNF-α, IL-6, and IL-1β in serum and ACTH and cortisol in plasma were tested by Radioimmunoassay. The results showed that both QLY and WLY can reduce the score of pain ($P<0.01$) and swelling of ankle ($P<0.01$), can resist the infiltration of inflammatory cells, reduce newly capillary and pannus formation ($P<0.05$, 0.01), and can decrease the level of TNF-α($P<0.01$- 0.05). However, both of the formula showed some differences on the effect. WLY had obvious influence on reducing the level of ACTH and cortisol at time of 6:00 am, 12:00 am, and 24:00 pm ($P<0.05$, $P<0.01$). While QLY could increase the level of cortisol at 18:00 pm ($P<0.05$) and decrease at 6:00 am ($P<0.05$), the formula maintained the circadian rhythm of cortisol. Also QLY can induce or adjust the circadian rhythms of IL-β,IL-6 and TNF-α, while WLY can not. Our results suggest that QLY and WLY, with different property in herbal medicine, can make different effects in CIA rats by changing the level and pattern of ACTH, cortisol, IL-1β,IL-6, and TNF-α.
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133 Phytochemicals in the California avocado: preliminary evidence for inhibition of prostate cancer cell growth
Qing-Yi Lu, Qifeng Zhang, Vay Liang Go, David Heber
Presenting Author: Qing-Yi Lu (UCLA School of Medicine)
Avocado has been considered primarily a source of monounsaturated fats in the diet. However, little is known regarding its other phytochemical contents. The yellow-green color of the avocado prompted us to initiate additional studies of the avocado composition. The purposes of the study were to determine concentrations of carotenoids, retinol, and vitamin E in the avocado and to examine the potential of avocado extract to inhibit PC-3 prostate cancer cell growth. Carotenoids and fat-soluble vitamins were determined in the California Hass avocado (Mission Produce Inc., Oxnard, CA) using high-pressure liquid chromatography. Seasonal and sample-to-sample variations were measured and considered in this analysis. Prostate cancer cells were quantitated using 3H-thymidine after 72-hrs incubation. Avocados were found to contain more lutein (293 Ug/100g) than any other fruit, and to contain significant amounts of vitamin E (3205 ug/100g), as well. Avocado extract was shown to reduce PC-3 prostate cancer cell line growth by 18% and 44 % at concentrations of 83 and 250 ug/ml extract, respectively. Our study indicates that avocado is a fruit which may contribute in the potential cancer prevention and is more than simply a source of monounsaturated fat.
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134 Black cohosh *(cimicifuga racemosa)* does not have any estrogenic activity
HA Oketch-Rabah, I Mehemi, MS Tsai, E Atlas, E Kennelly, P Nuntanakorn, F Kronenberg, R Lupu
Presenting Author: HA Oketch-Rabah
Purpose: Black cohosh (BC) is currently being taken by many American women to alleviate menopausal symptoms such as hot flashes. Estrogen, the primary treatment for hot flashes, is not recommended for women at high risk for breast cancer, or for breast cancer patients and survivors. The mechanism by which BC reduces the frequency of hot flashes is still unknown. The goal of our studies was to determine whether there is any estrogenic-like activity in extracts derived from BC, and to determine the safety of BC for women who should not take or choose not to take estrogen. The black cohosh extract used in these studies is currently under clinical trial for hot flashes at Columbia University. **Methods:** a) Extract preparation: Extracts from BC roots and rhizomes were made in hexane (BC1), ethyl acetate (BC2) and water (BC3), by sequential solvent-solvent partitioning of an aqueous-methanol BC crude extract, b) Ishikawa cell assay: Estrogenic activity was determined by the Ishikawa cell assay that measures the estrogenic activity of a compound (s) by inducing endogenous alkaline phosphatase (AP) enzyme activity, c) Transcriptional activation assay: At the molecular level, we tested the ability of the BC extracts to modulate the estrogen receptor (ER) function as evaluated using the ERE-luciferase reporter assay, d) RNAse Protection assays (RPA): We assess the extracts' ability to regulate the mRNA expression of E2-regulated genes, ER-α, PgR and pS2. These genes are regulated by synthetic estrogen and by genistein in ER-expressing breast cancer cells, e) Anchorage-dependent and -independent growth assays: We tested the ability of extracts derived from BC to induce the growth of breast cancer cells in anchorage-dependent and -independent assay. ER+ breast cancer cells were used for these assays. The assays were performed using concentrations of 0-20mg/ml. **Results:** In the Ishikawa cells based assay, the BC extracts did not enhance the AP activity, indicating no estrogenic activity. In addition, none of the BC extracts induced either ERE activity or regulation of known estrogen-regulated genes. By contrast, the synthetic estradiol (E2) significantly increased ERE-activity and regulated the expression of E2-regulated genes in breast cancer cells that express ER. Finally, we demonstrated that neither extract of BC had estrogenic effect on the growth of ER-expressing breast cancer cells. Control E2 significantly induced cell proliferation and colony formation. **Conclusion:** All of our results determine that no estrogenic activity is present in any of the BC extracts tested in our laboratory. Therefore, BC roots and rhizomes appear safe for use as an herbal remedy for the treatment of hot flashes in women for whom estrogen therapy presents a risk.
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135 Electroacupuncture stimulation of hindlimb acupoints induces expression of c-fos protein in the brain pathways
Xiao-Xue Zhang, Sheng-Xing Ma, Xi-Yan Li
Presenting Author: Sheng-Xing Ma (UCLA School of Medicine)
Purpose: The expression of immediate early gene, c-fos, has been used to map the distribution of brain neurons activated by stimulation, and Fos-like immunoreactivity (FLI) serves as a marker of neuronal activity to trace the neuronal pathway. We have recently observed that neuronal nitric oxide synthase expression is predominantly increased in the gracile nucleus with electroacupuncture (EA) stimulation of hindlimb acupuncture points (acupoints) in rats. The gracile nucleus receives peripheral somatosensory afferent inputs projecting from the hindlimb. Gracile-thalamic pathway plays an important role in the central modulation of somatosympathetic and cardiovascular functions. In the present study we examined the influence of EA stimulation on the expression of FLI in the brainstem, thalamus and cortex by using immunohistochemical technique. **Methods:** Low-frequency EA stimulation (3 Hz) was applied to the hindlimb acupoints, Jinggu and Shugu (BL 64-65), in rats anesthetized with ketamine. Rats in the sham-treated group received surgery and EA needles were placed into the acupoints without performing the stimulation. After 2 hours stimulation and sham-treatment, the animals were perfused with 4% paraformaldehyde. Sections of rat brain were examined by immunolabeling with a polyclonal antibody directed against c-fos. **Results:** Unilateral EA stimulation of BL 64-65 caused increases in c-fos immunostained cells (133±32% [mean±SE]) in the ipsilateral gracile nucleus, and (74±28%) in the contralateral sides compared with sham-treated rats ($P<0.05$, $n=4$). c-Fos immunostaining

Appendix V

BLACK COHOSH *Actaea racemosa* L., HAS NO ESTROGENIC ACTIVITY

Authors: Hellen A. Oketch-Rabah , Tsai MS,...and Lupu, R

ABSTRACT

Black cohosh (BC) is a remedy currently being taken by many women as an alternative to estrogen (E2) in order to alleviate menopausal symptoms, such as hot flashes. The goal of the present study is to determine whether BC extracts contain E2 activity, and to evaluate the safety of BC for those who are breast cancer patients or are at high risk of developing breast cancer. Extracts (hexane, ethyl acetate, and aqueous) were prepared from BC by sequential solvent partitioning. The ability of BC to modulate estrogen receptor (ER) function, to regulate E2-responsive gene expression, and to induce growth of ER-positive breast cancer cells was each tested. We found that no E2 activity was detected in BC extracts, as determined by the Ishikawa cell assay, which determines whether BC induces E2-regulated endogenous alkaline phosphatase activity. Using the ERE-luciferase reporter assays, we found that the BC extracts did not modulate ER function by transactivating the estrogen-responsive-element (ERE). We also found that the BC extracts did not regulate the expression of E2-responsive genes as determined by the RNAse protection assays. Furthermore, none of the BC extracts had an E2 effect on the proliferation and colony formation of ER-expressing breast cancer cells, such as MCF-7 and T47D cells, as determined by both anchorage-dependent and -independent growth assays. Our results demonstrate that extracts derived from BC do not contain E2-like activity. Therefore, in women for whom E2 therapy presents a risk, BC roots and rhizomes appear safe for use as an herbal remedy for the treatment of menopausal symptoms such as hot flashes

Appendix VI

Transforming Growth Factor-β1 Regulates the Proliferation of Estrogen Receptor Positive Mammary Epithelial Cells

Authors: Hellen A. Oketch-Rabah, Kenneth Ewan, Shraddha Ravani, Shyamala G. and Mary Helen Barcellos-Hoff Department of Cell and Molecular Biology, Ernest Orlando Lawrence Berkeley National Laboratory , Berkeley, California 94720

ABSTRACT

Estradiol (E2) signaling through ER-α (one of the isoforms of ER) plays a central role in mammary gland epithelial proliferation. In the ER knockout mouse, a lack of ductal outgrowth has been observed, further confirming that ER+ve cells are necessary for the proliferation that is required to facilitate ductal elongation in the mammary glands. However, a variety of recent studies have shown that estrogen receptor positive (ER+ve) cells rarely proliferate. It is mainly the ER-ve cells that proliferate and stain with proliferation markers and these proliferating ER-ve cells are frequently located next to ER+ve cells. Thus it appears that the ER+ve cells, in some way, regulates the proliferation of ER-ve cells by a paracrine mechanism.

Studies carried out in our lab showed that at estrus when cell proliferation peaks, nearly all ER+ve cells co-localize with intense active TGF-β1 staining, consistent with their non-proliferative status. We therefore hypothesized that TGF-β1 restrains the mammary epithelial cells from proliferating and that this in turn regulates mammary epithelial proliferative response to estrogen.

To discern the possible role of TGF-β1, epithelial cells of *Tgfβ1 1* +/+ and *Tgfβ1 1* +/- Balb/c mice mammary glands were scored for ER and Ki-67 immunoreactivity and the frequency of their co-localization at different phases of the estrus cycle representing quiescent stage of development and stages of proliferation. There were no proliferating ER+ve cells at proestrus. However, at estrus the frequency of ER+ve cells that also stain for Ki67 respectively in *Tgfβ1* +/+ and *Tgfβ1* +/- epithelium is 1.0% and 2.0% of the total cells. At diestrus in the *Tgfβ1* +/+ epithelium, there is a 3-fold decrease lowering the frequency to 0.2%, while in the *Tgfβ1* +/- mice the proportion of proliferating ER+ cells remains elevated (2.8%) during diestrus.

Previous data in the C57Bl/6-sv 129 mixed background also show increased proliferation of ER+ve mammary epithelial cells at estrus. This resulted in a significant increased frequency of ER+ve cells in the *Tgfβ1* +/- mammary epithelium compared to *Tgfβ1*+/+ mice. However,in Balb/c mice this change was not observed.

In conclusion our data shows that reduced levels of TGF-β1 leads to increased proliferation of ER+ve epithelial cells consistent with our hypothesis that TGF-β1 restrains the proliferation of ER+ve mammary epithelial cells in mouse mammary glands.

Appendix VII

Transforming Growth Factor-β1 Regulates the Proliferation of Mammary Epithelial Cells

Authors: [1]Hellen A. Oketch-Rabah*, [1]Kenneth B. Ewan, and Mary [1]Helen Barcellos-Hoff
[1]Department of Cell and Molecular Biology, Ernest Orlando Lawrence Berkeley National Laboratory , Berkeley, California 94720
*Presenting

ABSTRACT: Estradiol (E2) signaling through ER-α (one of the isoforms of ER) plays a central role in mammary gland epithelial proliferation. In the ER knockout mouse, a lack of ductal outgrowth has been observed, further confirming that ER+ve cells are necessary for the roliferation that is required to facilitate ductal elongation in the mammary glands. However, a variety of recent studies have shown that estrogen receptor positive (ER+ve) cells rarely proliferate. It is mainly the ER-ve cells that proliferate and stain with proliferation markers and these proliferating ER-ve cells are frequently located next to ER+ve cells. Thus it appears that the ER+ve cells, in some way, regulates the proliferation of ER-ve cells by a paracrine mechanism. Studies carried out in our lab showed that at estrus when cell proliferation peaks nearly all ER+ve cells co-localize with intense active TGF-β1 staining, consistent with their non-proliferative status. We therefore hypothesized that TGF-β1 restrains the mammary epithelial cells from proliferating and that this in turn regulates mammary epithelial proliferative response to estrogen.To discern the possible role of TGF-β1, epithelial cells of *Tgfβ1 1* +/+ and *Tgfβ1 1* +/- Balb/c mice mammary glands were scored for ER and Ki-67 immunoreactivity and the frequency of their co-localization at different phases of the estrus cycle representing quiescent stage of development and stages of proliferation. There were no proliferating ER+ve cells at proestrus. However, at estrus the frequency of ER+ve cells that also stain for Ki67 respectively in *Tgfβ1* +/+ and *Tgfβ1* +/- epithelium is 1.5% and 3.5% of the total cells. At diestrus in the *Tgfβ1* +/+ epithelium there is a 3-fold decrease bringing the frequency to 0.5%, while in the *Tgfβ1* +/- mice the proportion of proliferating ER+ cells remains high during diestrus (2.8%).
Previous data in the C57Bl/6-sv 129 mixed background also show increased proliferation of ER+ve mammary epithelial cells at estrus. This resulted in a significant increased frequency of ER+ve cells in the *Tgfβ1* +/- mammary epithelium compared to *Tgfβ1*+/+ mice. However,in Balb/c mice this change was not observed.
In conclusion our data shows that reduced levels of TGF-β1 leads increased proliferation of ER+ve epithelial cells consistent with our hypothesis that TGF-β1 restrains the proliferation of ER+ve mammary epithelial cells in mouse mammary glands.

Appendix VIII

Statement of Work (SOW2) for (2002/3)

The Role of TFG-β1 in the Regulation of Estrogen Receptor During Mouse Mammary Development and Carcinogenesis

It is also well known that estradiol (E2) signaling through ER-α (one of the isoforms of ER) plays a central role in mammary epithelial cell proliferation. However a variety of recent studies have shown that estrogen receptor positive (ER+) cells do not proliferate. ER- cells usually proliferate and stain with proliferation markers such as Ki67 but are frequently located next to ER+ cells. Thus, although ER+ cells do not proliferate, they are necessary for proliferation, as shown by the lack of ductal outgrowth in the ER knockout mouse, and appear to regulate the proliferation of ER- cells via a paracrine mechanism.

Transforming growth factorβ1 (TGF-β) is the most potent inhibitor of human and mouse mammary epithelial cell proliferation known. Studies in our lab have shown that at estrus when there is a high rate of cell proliferation, nearly all ER+ cells co-localize with intense TGF-β staining, consistent with their non-proliferative status. It appear that TGF-β acts as a brake restraining the ER+ cells from proliferating while at the same time the ER+ cells, in response to hormonal stimulation by estrogen (E2), send out a signal to the ER- cells to proliferate. This hypothesis seems plausible given that when TGFβ1 level is reduced (as is the case in the *Tgfβ1* (+/-), ER+ cells proliferate more as evidence by the increase in the population of ER+ positive cells compared to the same population in the *Tgf-β1* (+/+) mice. Understanding the role that TGFβ plays in the proliferation of ER+ cells is important because it is known that ER+ breast cancer can progress to more aggressive ER- negative cancer that is by its nature anti estrogen resistant and is more likely to become a deadly metastatic disease. The mechanism by which breast cancer progresses from the E2-dependent phenotype to the E2-independent one is not yet fully understood and yet it is important clinically as it would help identify possible targets of intervention in the control of or halting breast cancer progression.

Specifically understanding the role of TGβ in this process may unveil how TGFβ could be targeted in the control of breast cancer since it is well known that increased TGFβ activity is associated with breast cancer progression (5) and can functionally mediate metastatic disease (6-8). The project will make use of *Tgf-β1* (+/-) Balb/c mice as a model and will include immunohistochemisrty studies, protein analysis and primary culture of mouse mammary tissue.

The specific objectives in this project are:

1) To substantiate the role of TGF-β in regulation of ER during mammary development I will determine the frequency of ER+ cells and the level of ER as a function of TGF-β activity in Balb/c mice *Tgf-β1* (+/-) and *Tgf-β1* (+/+) mice and compare these data to those previously obtained in the C57bl/129SV.

2) To determine whether TFGβ suppresses ER we will use primary mouse mammary epithelial and human breast cell cultures.

[CANCER RESEARCH 60, 5603–5607, October 15, 2

Advances in Brief

Appendix IX

Expression and Function of CYR61, an Angiogenic Factor, in Breast Cancer Cell Lines and Tumor Biopsies[1]

Miaw-Sheue Tsai, Ann E. Hornby,[2] Johnathon Lakins,[3] and Ruth Lupu[4]

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Abstract

We have previously shown that expression of heregulin (HRG) is closely correlated with breast cancer progression. We have subsequently isolated Cyr61, a ligand for the $\alpha_v\beta_3$ integrin that is differentially expressed in HRG-positive cells, and have shown that it is expressed in all of the invasive and metastatic breast cancer cell lines tested. Preliminary evaluation of Cyr61 expression in breast tumor biopsies revealed expression of Cyr61 in about 30% of invasive breast carcinomas. Significantly, we demonstrated that Cyr61 is a downstream effector of HRG action, because a Cyr61-neutralizing antibody abolished the ability of HRG-expressing cells to migrate *in vitro*. Furthermore, we have shown that HRG-expressing cells denote higher levels of $\alpha_v\beta_3$ expression, and we have established that Cyr61 action is mediated, at least in part, through its receptor $\alpha_v\beta_3$, because a functional blocking antibody of the $\alpha_v\beta_3$ blocked the Matrigel outgrowth of HRG-expressing cells. These results strongly suggest that Cyr61 is necessary for HRG-mediated chemomigration and that Cyr61 plays a functional role in breast cancer progression, possibly through its interactions with the $\alpha_v\beta_3$ receptor.

Introduction

Many E2[5]-dependent and antiestrogen-responsive breast tumors spontaneously progress to an E2-independent and antiestrogen-resistant phenotype, becoming deadly metastatic diseases. The mechanism by which breast cancer appears to progress from an E2-dependent to an E2-independent phenotype is still under investigation. We have shown that expression of HRG, an activator of *erb*B-2/-3/-4 receptor signaling pathways, is closely associated with an invasive breast cancer phenotype (1). Furthermore, we demonstrated that HRG induces breast cancer progression, as determined by loss of ER function and response, tumorigenicity (2), invasion (3), and metastasis (4). It has been hypothesized that HRG induces activation of the *erb*B signaling pathways, leading to regulation of downstream genes that regulate and control cancer progression. Therefore, to develop effective targeted therapies, it is important to identify gene(s) directly involved in HRG-induced breast cancer aggressiveness.

With this in mind, we have isolated and identified the human homologue of a mouse immediate-early response gene, *Cyr61*, differentially expressed in ER-negative, HRG-positive breast cancer cells. Cyr61 is a secreted cysteine-rich protein that is associated with the cell surface and the extracellular matrix (5). Cyr61 mediates cell adhesion, migration, and angiogenesis (6, 7). In this report, we establish that Cyr61 is coexpressed with HRG in all of the metastatic breast cancer cell lines tested, its expression is inversely correlated with ER expression, and it is associated with HRG-induced breast cancer chemomigration and metastasis, possibly through interactions with the $\alpha_v\beta_3$ integrin receptor. Furthermore, we establish that Cyr61 was expressed in about 30% of invasive breast cancer tumor biopsies, implying a possible role in breast cancer progression.

Materials and Methods

Cells and Cell Culture

Breast cancer cell lines were obtained from the American Type Culture Collection and routinely cultured in phenol red-containing improved MEM supplemented with 5% (v/v) fetal bovine serum and 2 mM L-glutamine at 37°C in a humidified atmosphere with 5% CO_2, unless otherwise specified.

Plasmids and Generation of Riboprobes

A Cyr61 riboprobe plasmid was constructed by cloning a PCR fragment of Cyr61 cDNA into the pCRII TA cloning vector (Invitrogen). The sequence of primers used to generate the Cyr61 fragments was as follows: (*a*) forward primer, 5′-TGTGGAACTGGTATCTCCACACGA-3′ (nucleotides 727–750); and (*b*) reverse primer, 5′-TCTTTTCACTGAATATAAAATTAAAA-3′ (nucleotides 1739–1764). The Cyr61 riboprobe construct was sequenced using Sequenase v.2.0 with ^{35}S-labeled dCTP. Radioactive riboprobe was prepared by linearizing the plasmid with the restriction enzyme *Dde*I, which generated a 524-bp fragment, and followed by reverse transcription *in vitro* using the SP6 RNA polymerase in the presence of [^{32}P]UTP. The riboprobe plasmid of GAPDH was kindly provided by Dr. Francis Kern (University of Alabama, Birmingham, AL). Radiolabeled GAPDH riboprobe was generated using T7 RNA polymerase as described above, except that it was linearized by the restriction enzyme *Bam*HI.

RNase Protection Assay

Total RNA was extracted by Tripure isolation solution (Roche Molecular Biochemicals) and quantified by spectrophotometry. RNA (30 μg) was hybridized with 100,000 cpm of ^{32}P-labeled Cyr61 riboprobe for 12–16 h at 50°C. ^{32}P-labeled GAPDH riboprobe (10,000 cpm) was added to each sample as an internal control. Hybridized RNA samples were digested with 25 μg of RNase A for 30 min at 28°C. The reaction was terminated by incubating with proteinase K (250 μg/ml) and 0.5% SDS for 15 min at 37°C. After phenol extraction, RNA samples were coprecipitated with 10 μg of yeast tRNA in absolute ethanol. RNA was redissolved in a denaturing loading buffer and resolved by electrophoresis on a 6% polyacrylamide-urea gel. Protected fragments of Cyr61 (305 bp) and GAPDH (100 bp) were visualized by autoradiography. [^{32}P]dCTP-end-labeled pBR322/*Msp*I (New England Biolabs) was used as a molecular weight marker.

Western Blot Analysis

Cyr61 Present in Conditioned Media. Subconfluent human breast cancer cells were maintained in serum-free media for 3–4 days. The conditioned media were collected, and the Cyr61 protein was purified by heparin affinity

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[5] The abbreviations used are: E2, estradiol; Tam, tamoxifen; ER, estrogen receptor; HRG, heregulin; GAPDH, glyceraldehyde-3-phospate dehydrogenase.

chromatography. The column was washed with 0.3–0.6 M NaCl in 10 mM Tris-HCl (pH 7.5). The Cyr61 protein was eluted at 0.9 M NaCl and desalted by PD-10 Sephadex G25M columns (Amersham-Pharmacia). The eluted fractions were concentrated (10×) and resolved by 12% Tris-glycine SDS-PAGE. The separated proteins were electroblotted onto a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham-Pharmacia). The blotted membrane was blocked overnight at 4°C with 5% (w/v) BSA in Tris-buffered saline containing 0.5% Tween 20 (TBST) and incubated with the rabbit anti-Cyr61 polyclonal antibody (1:5,000 dilution) for 1 h at room temperature. After three washes with TBST, the blot was incubated with a 1:10,000 dilution of horseradish peroxidase-linked donkey antirabbit IgG secondary antibody. The Cyr61 protein was detected by the enhanced chemiluminescence reaction using Hyperfilm (Amersham-Pharmacia).

Cyr61 Present in Breast Cancer Tumor Specimens. Breast tumor specimens were lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM DTT] with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride). Protein concentration was determined by a Micro BCA detection reagent kit (Pierce). Equal amounts of proteins were loaded and separated by SDS-PAGE followed by Western blot analysis as described above.

Immunohistochemical Staining

Formalin-fixed paraffin-embedded breast tumor sections were deparaffinized in xylene and hydrated in a graded alcohol series. Slides were quenched for endogenous peroxidase activity in the presence of 0.3% H_2O_2 for 30 min and blocked with 10% (v/v) horse serum for 30 min. Slides were then incubated with a polyclonal anti-Cyr61 antibody (1:5000) overnight at 4°C. The sections were washed in PBS before the incubation with a biotinylated antirabbit IgG secondary (1:200) antibody for 30 min. The sections were then incubated with an avidin-biotin complex (VECTASTAIN Elite ABC reagent; Vector Laboratories) for 30 min, and the reaction was developed in the presence of hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride. The slides were counterstained with hematoxylin solution and mounted with the aqueous Crystal mount media.

Chemomigration and Chemotaxis Assays

Boyden chamber chemomigration assays were performed using a 48-well chemotaxis chamber (Neuro Probe). Breast cancer cells (20,000 cells/well) were plated onto the upper chambers in triplicate or quadruplicate onto a 12 μm polycarbonate filter membrane coated with collagen (Becton Dickinson). The conditioned media derived from NIH3T3 fibroblast culture was used as a chemoattractant in the lower chambers. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 16 h. After the incubation, the membrane was removed from the chamber. The cells on the top surface were removed, and the cells on the bottom side of the membrane were fixed in methanol and stained with a Diff-Quick Stain kit. Membranes were then mounted onto glass slides, and the cells that migrated through the pores to the opposite side of the membrane (bottom side) were quantified using a light microscope.

Matrigel Outgrowth Assay

Cells (5,000 cells/well) were mixed with 150 μl of Matrigel (Becton Dickinson) and plated in triplicate onto the Matrigel-coated 12-well plates for 1 h at 37°C. Cells were then cultured in the media containing the indicated concentrations of antibodies for 7–10 days. The pattern of the cells' outgrowth in Matrigel matrix was examined and photographed using a phase-contrast microscope.

Results and Discussion

Cyr61 Is Differentially Expressed in HRG-positive *versus* HRG-negative Breast Cancer Cell Lines. We have demonstrated that expression of HRG is highly associated with aggressive progression of breast cancers to hormone independence, antiestrogen resistance, invasion, and metastasis (2–4). To identify genes that were involved in the HRG induction of breast cancer progression, a number of genes were isolated and cloned by differential expression in MDA-MB-231 HRG-expressing cells. Sequence and homology analyses indicated that one of the genes is the human homologue of a mouse immediate-early response gene, *Cyr61*. Cyr61 was highly and selectively expressed in MCF-7/HRG [MCF-7/HRG (HRG-transfected MCF-7) clones, *e.g.*, T2, T6, T7, and T8] but was nearly undetected in MCF-7/V cells (vector-transfected MCF-7 cells). A 5–25-fold increase in the Cyr61 mRNA level was observed in MCF-7/HRG cells as compared with MCF-7/V cells. HRG-positive MDA-MB-231 cells also expressed high levels of Cyr61 (Fig. 1*A*). To determine whether the protein was also selectively expressed, we performed Western blot analysis and immunohistochemistry using an anti-Cyr61 polyclonal antibody. As shown in Fig. 1*B*, Cyr61 protein expression was observed in the MCF-7/HRG cells but not in the vector control cells. These studies were performed using cells cultured under serum-depleted conditions.

In addition, immunohistochemical staining was performed on paraffin sections of MCF-7/HRG and MCF-7/V tumors formed in xenografted athymic nude mice. These tumors were observed as a mixture of solid, trabecular, and tubular patterns. Irregular gland formation and occasional well-formed lumen were present. These features, as well as the heterogeneity and variety of histological patterns, resemble those observed in mammary infiltrating ductal carcinoma known as no special type (Fig. 1*A*). Similar features were observed in all of the tumors examined. As can be seen in Fig. 1*C*, Cyr61 expression was very predominant in MCF-7/HRG-derived tumors (*right panel*). Expression of the Cyr61 protein was localized to the cytoplasm of the tumor cells, whereas only a weak staining of Cyr61 was observed in tumors derived from MCF-7/V cells supplemented with E2 (*left panel*). Once again, our data demonstrate a differential expression of Cyr61 in HRG-expressing cells.

We mapped the human *Cyr61* gene to chromosome 1p (data not shown), consistent with previous studies showing the localization of Cyr61 to chromosome 1p22.3 (8, 9). Abnormalities of chromosome 1p have correlated with ER negativity and a poor prognosis in breast cancer (10) and other malignancies (11–13).

It has been shown that murine Cyr61 is regulated by 12-*O*-tetradecanoylphorbol-13-acetate in the liver (14), as well as by E2 and Tam in the uterus (15). We have shown that the human homologue of Cyr61 is regulated by E2 and several antiestrogens including Tam and ICI 182,780 in ER-positive breast cancer cells.[6] The induction of Cyr61 was most significant in MCF-7 cells [up to a 10–12-fold increase by 6 h of treatment with E2 (10^{-9} M) or 3 h of treatment with ICI 182,780 (10^{-7} M)] and to about a 5–6-fold increase with Tam (10^{-7} M). On the other hand, the up-regulation of Cyr61 was not significant in HRG-expressing cells, with an increase of only 1.5–2-fold by any of the treatments (data not shown). These results are consistent with our published data demonstrating that HRG promotes an estrogen-independent phenotype and that HRG blocks ER function resulting in MCF-7/HRG cells that fail to respond to E2 and the consequential inability of E2 to induce the expression of E2-regulated genes.

Cyr61 Is Overexpressed in HRG-positive, ER-negative Breast Cancer Cell Lines. To assess whether up-regulation of Cyr61 expression was a result of HRG overexpression in MCF-7/HRG cells or whether it was a common theme occurring in breast cancer cells, we examined its expression in many human breast cancer cell lines. Basal level of Cyr61 expression was measured in cells cultured under serum-depleted conditions to prevent the influence of E2 on Cyr61

[6] M. S. Tsai, E. Gilad, M. Cardillo, and R. Lupu. Heregulin (HRG) promotes tumor formation, manuscript in preparation.

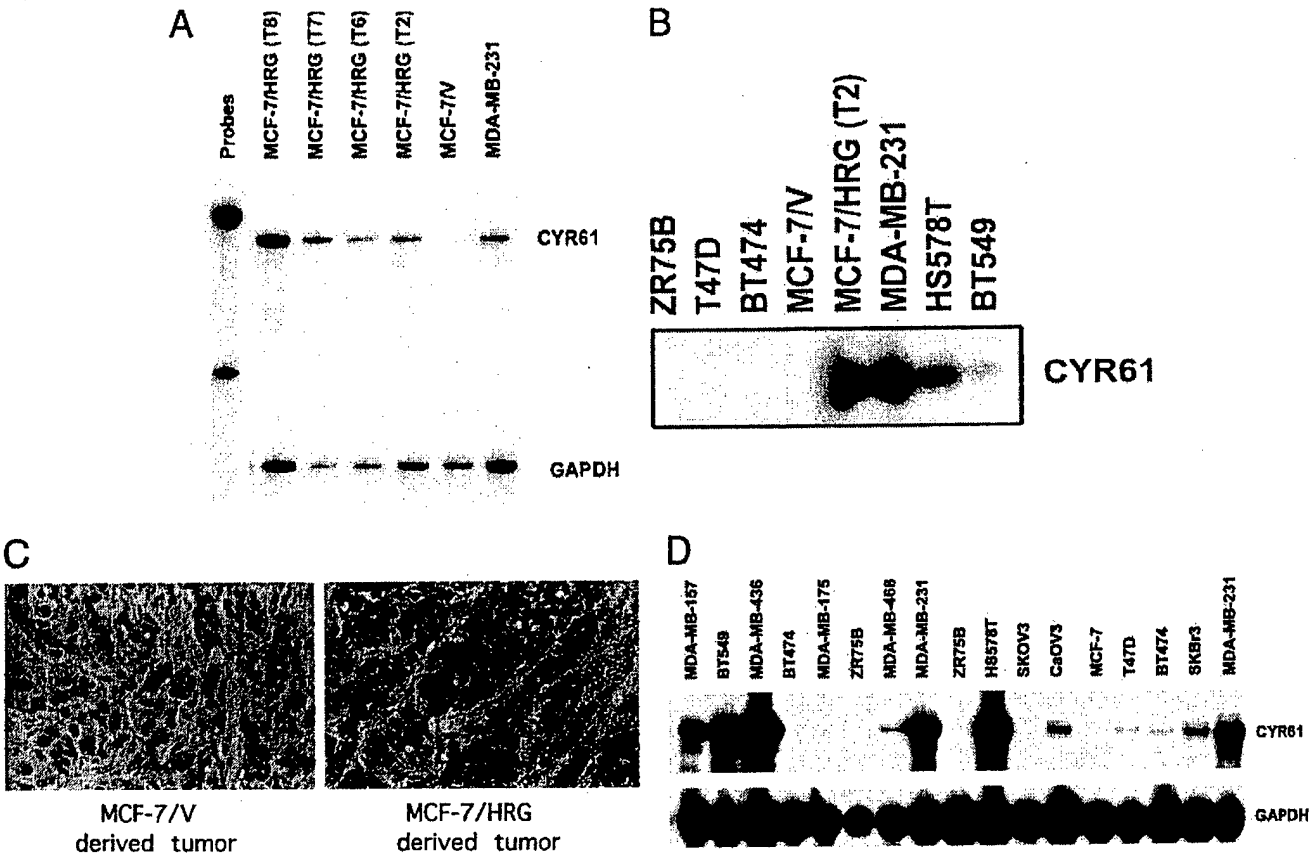

Fig. 1. Expression of Cyr61 in breast cancer cells. *A*, subconfluent MCF-7/HRG and MCF-7/V cells were maintained in serum-depleted conditions for 3 days. Total RNA was isolated, and 30 μg of RNA were analyzed by RNase protection assay. RNA from MDA-MB-231 cells was used as a positive control for Cyr61 expression. The GAPDH probe was used as an internal control for RNA loading. *B*, subconfluent breast cancer cell lines were cultured in serum-free media. Conditioned media were collected after 3 days, and heparin column chromatography was performed. The Cyr61 protein was eluted and analyzed by Western blotting analysis as described in "Materials and Methods." *C*, immunohistochemical analysis was performed as described in "Materials and Methods" for MCF-7/V- and MCF-7/HRG-derived tumor sections. *D*, breast cancer cells were cultured as described in *A*, and total RNA was isolated and analyzed by RNase protection assay.

expression. As shown in Fig. 1, *B* and *D*, a tight correlation between Cyr61 mRNA and protein expression exists in all of the cell lines tested. Cyr61 is highly expressed in MDA-MB-231, HS578T, BT549, and MCF-7/HRG cells, all of which are HRG-expressing and ER-negative cells, but it is low or undetectable in cells that do not express HRG and are ER-positive, including MCF-7, ZR75B, T47D, and BT474 cells. These studies were performed by RNase protection assays and by Western blot analysis in which a secreted M_r 45,000 protein derived from conditioned media was detected using an anti-Cyr61 polyclonal antibody.

Our data indicate that a high level of Cyr61 expression correlates with HRG expression and inversely correlates with ER expression, response to E2, and sensitivity to antiestrogens (16). Moreover, the expression of Cyr61 strongly correlates with vimentin expression, a known marker for invasiveness (17), and is associated with the ability of breast cancer cells to invade *in vitro* and metastasize *in vivo*. On the other hand, low to undetectable levels of Cyr61 expression were seen only in the HRG-negative, ER-positive, E2-dependent, antiestrogen-sensitive breast cancer cells. These data are summarized in Table 1. Taken together, these data show that Cyr61 expression is associated with HRG expression and is apparently linked to breast cancer progression. Because *Cyr61* is an early response gene, it could be argued that its expression would be up-regulated in rapidly proliferating cells. Thus, it is critical to establish that up-regulation of Cyr61 in MCF-7/HRG cells is not attributable to a proliferative advantage of these cells. Cell cycle analysis by flow cytometry demonstrated that no differences in cell cycle distribution were observed between the MCF-7/HRG cells and the parental MCF-7 cells (18).

Cyr61 Is Expressed in about 30% of Breast Tumor Biopsies. To determine whether expression of Cyr61 may have clinical relevance in breast cancer, its expression in biopsies was determined. A pilot study was performed using Western blot analysis on proteins extracted from paraffin sections. Forty percent (4 of 10) of the tumor specimens, all of which were ER-negative invasive breast carcinomas, showed high expression of the Cyr61 protein (Fig. 2*A*). Total cell lysates of MDA-MB-231 and MCF-7 were used as positive and negative controls, respectively. It is noteworthy that Cyr61 protein expression was low in cell lysates of MDA-MB-231, because Cyr61 is mostly secreted to the cultured media. Additional studies revealed that Cyr61 was detected in about 30% of breast tumor specimens (n = 55) by immunohistochemistry (Fig. 2*B*). Cyr61 staining was demonstrated to be specific because it was completely blocked in the presence of excess recombinant Cyr61 protein (data not shown). No staining was observed in normal components of the biopsies. These data suggest that in at least 30% of these tumors, Cyr61 may be required for survival; therefore, it may be strongly implicated in breast cancer progression.

An Anti-Cyr61-Neutralizing Antibody Blocks Chemomigration of MCF-7/HRG Cells. To demonstrate that Cyr61 is a direct downstream regulator of HRG action, studies were performed using a Cyr61-neutralizing antibody [Refs. 5–7; kindly provided by Dr. Lester F. Lau (University of Illinois, Chicago, IL)]. For these studies, we used MCF-7/HRG cells, which have been shown to migrate through collagen in a Boyden chamber assay (as shown below). Cells were treated with increasing concentrations of the antibody, and the ability of the cells to migrate *in vitro* was assessed. The anti-Cyr61-neutralizing antibody inhibited migration of MCF-7/HRG cells in a dose-

Table 1 *Expression of Cyr61 in breast cancer cell lines*[a]

Cell line	Cyr61	HRG	ER	Invasive *in vitro*	Metastatic *in vivo*	$\alpha_v\beta_3$[b]
MCF-7	−	−	++++	−[c]	−	+/−
T47D	−	−	++	−[c]	−	−
BT474	−	−	++	−[c]	−	−
MDA-MB-175	−	+/−[d]	+	−[c]	−	ND
ZR75B	−	+/−[d]	+	−[c]	−	−
MDA-MB-468	+	−	−	+[e]	−	−
SKBR-3	+	−	−	+[e]	−	−
MDA-MB-157	++	++	−	+	ND	ND
MDA-MB-436	+++	+++	−	+++	ND	ND
BT-549	+++	+++	−	+++	+	ND
MDA-MB-231	++++	++++	−	++++	+	+++
MDA-MB-435	++++	++++	−	++++	+	+++
HS578T	++++	++++	−	++++	+	ND
MCF-7/HRG	+++	+++	−/+	+++	+	++

[a] − indicates no expression; the number of plus signs indicates the increase in expression.
[b] The $\alpha_v\beta_3$ integrin expression is based on results from Ref. 19 and our preliminary data. ND, not determined.
[c] Cells require E2 for invasion *in vitro* and growth *in vivo* and never metastasize *in vivo*.
[d] E2 induces expression of HRG.
[e] Cells require ligand (epidermal growth factor or HRG) to invade but never metastasize *in vivo*.

dependent manner (Fig. 3*A*). No effect was observed when a control IgG antibody was used under the same conditions and concentrations. Similar results were observed in other invasive, HRG-expressing breast cancer cells, such as MDA-MB-231, HS578T, and BT549 (data not shown). These studies suggest, for the first time, a possible association between the increase in Cyr61 expression and the invasive potential triggered by HRG. Additional studies are required to assess the direct association between Cyr61 and HRG and their joint action resulting in breast cancer progression. Of note, the anti-Cyr61-neutralizing antibody had no effect on MCF-7 cells. It is important to note that MCF-7 cells do not migrate through collagen.

The $\alpha_v\beta_3$ Integrin Receptor Is Involved in Cyr61-Mediated Breast Cancer Progression. Because Cyr61 was shown to be a ligand for the $\alpha_v\beta_3$ integrin (19), we speculated whether Cyr61 requires expression of $\alpha_v\beta_3$ for its action. Thus, we assessed the level of $\alpha_v\beta_3$ expression in MCF-7/HRG cells and showed that the level of $\alpha_v\beta_3$ was augmented in MCF-7/HRG cells compared with the MCF-7/V cells (data not shown), as determined by immunofluorescence staining using an anti-$\alpha_v\beta_3$ antibody on cultured cells. We then speculated that if the action of Cyr61 is mediated through the $\alpha_v\beta_3$ receptor, it is plausible that blockage of the $\alpha_v\beta_3$ integrin will modulate the growth characteristics of MCF-7/HRG cells. Thus, Matrigel outgrowth and migration studies were performed in the presence and absence of an anti-$\alpha_v\beta_3$ functional blocking antibody. We determined that this antibody specifically blocked the Matrigel outgrowth of HRG-expressing cells in a dose-dependent manner (Fig. 3*B*). No effects were observed when control IgG was used. Similar inhibitory effects of the anti-$\alpha_v\beta_3$ antibody were seen in HRG-positive MDA-MB-231 cells (data not shown).

The results indicated that the functional $\alpha_v\beta_3$ integrin is required for maintaining the invasive capacity of HRG-expressing cells, and that the aggressive phenotypes induced by HRG are mediated, in part if not entirely, by Cyr61 and its receptor, $\alpha_v\beta_3$ integrin. Because Cyr61, an angiogenic factor, and its receptor, $\alpha_v\beta_3$, are both induced

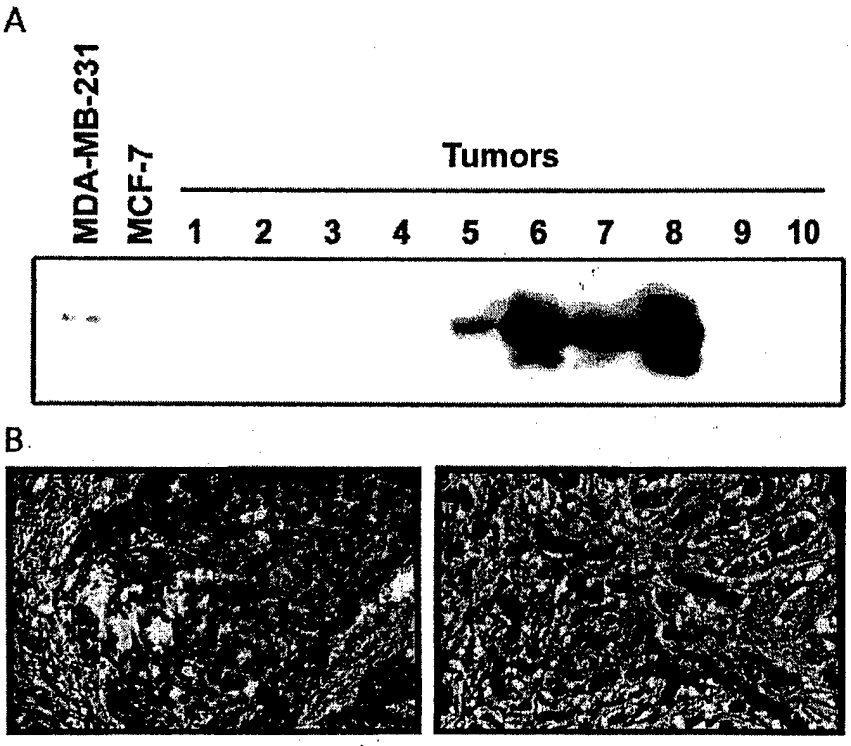

Fig. 2. Expression of Cyr61 in human breast tumor biopsies. *A*, human breast tumors were lysed in radioimmunoprecipitation assay buffer, and equal amounts of protein were resolved on a 4–20% gradient SDS-polyacrylamide gel. Western blotting analysis was performed as described in "Materials and Methods." Cell lysates of MCF-7 and MDA-MB-231 cells were used as negative and positive controls, respectively. *B*, immunohistochemical analysis of human breast carcinoma biopsies was performed as described in "Materials and Methods." Two representative microphotographs are shown for Cyr61 expression.

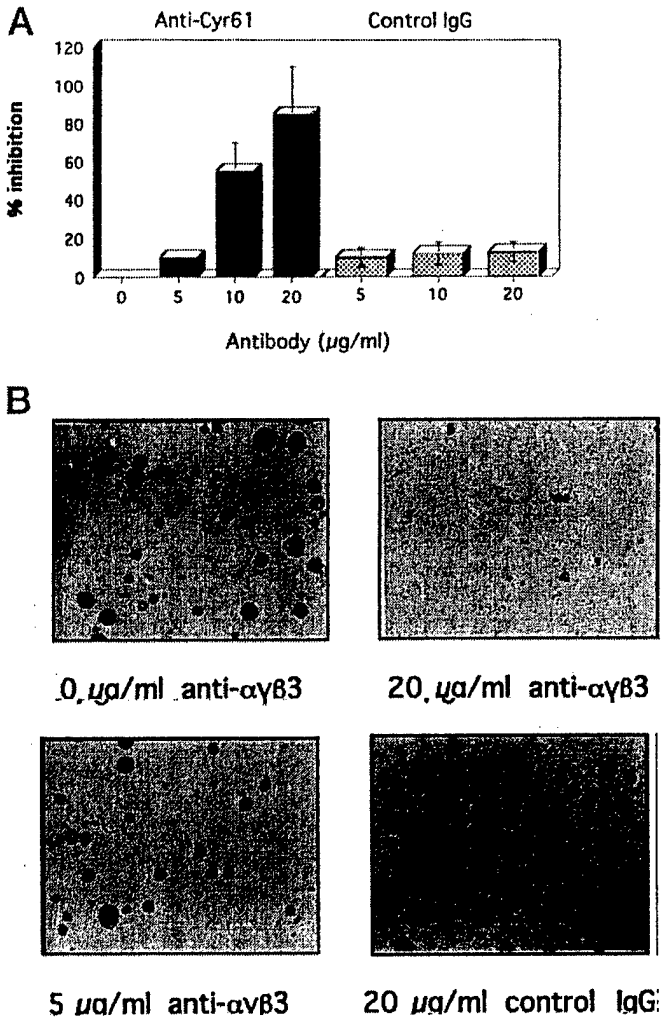

Fig. 3. *A*, blocking the invasive phenotypes of HRG-expressing cells by a Cyr61-neutralizing antibody. MCF-7/HRG cells were treated in the absence or presence of increasing concentrations (5, 10, and 20 μg/ml) of the anti-Cyr61 antibody or a control IgG for 16 h in the Boyden chamber assay. Chemomigration was measured based on the number of cells traversing collagen-coated filters. Data are the mean of triplicates from a representative experiment. SD was calculated for each data point. *B*, inhibited outgrowth of MCF-7/HRG cells by a functional blocking antibody of $\alpha_v\beta_3$. MCF-7/HRG cells were treated in the absence or presence of increasing concentrations of the anti-$\alpha_v\beta_3$ antibody (LM609; only the 5 and 20 μg/ml concentrations of antibody are shown) or a control IgG (20 μg/ml) in Matrigel outgrowth assay for 7 days. Outgrowth pattern was examined and photographed.

in the MCF-7/HRG cells, it is tempting to postulate that these factors are involved in the increased neovascularization that we have observed in the tumors formed by MCF-7/HRG cells in athymic nude mice.[6] The exact mechanism by which HRG promotes an aggressive breast cancer phenotype is still unknown. However, the identification of Cyr61 expression in breast cancer tumor progression is of great significance, especially because its receptor, the $\alpha_v\beta_3$ integrin, was recently shown to be a good prognostic indicator in breast cancer (20, 21). Studies are under way to determine whether ectopic expression of Cyr61 alone, in HRG-negative cells, is sufficient and/or necessary to confer some biological activities induced by HRG, such as loss of E2 response, acquisition of antiestrogen resistance, and chemomigration.

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